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LIPID PEROXIDE FORMATION AND PHOSPHOLIPID IN NORMAL AND TUMOR TISSUES*

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That homogenates of various tissues can produce lipid peroxides on aerobic incubation has repeatedly been reported (1-5). On the other hand, Shuster (6) found that homogenates of Ehrlich ascites tumor produces no thiobarbituric acid (TBA)-reacting substance (lipid peroxide) whatsoever on incubation or irradiation. In regenerating rat liver and bone marrow where cell division is actively taking place, peroxide values are also very low (7, 8). Also, the author has shown that the capability of peroxide formation is considerably decreased in livers of tumor-bearing rats and nearly all lost in tumor tissues, and that the peroxide formation is completely inhibited whether in normal or tumor tissues when ethylenediaminetetraacetic acid (EDTA) is added to the reaction mixture. Furthermore, it has been found that of the metals examined, zinc alone is effective in restoring the peroxide formation in EDTA-treated systems (9).

In the present study, homogenates of normal and tumor tissues were respectively fractionated into lipid and residue (protein) fractions and these fractions were examined for the activity of lipid peroxide formation. It was very interesting to observe that whereas neither the lipid nor residue fraction alone showed an appreciable TBA value, the combination of both fractions invariably showed a high peroxide value. This lipid fraction was then further fractionated and it was found that the tissues owe much of their peroxide forming activity to their phospholipids and especially to non-choline phospholipid fraction reported by Hevesy (11) although the addition of residue (protein) fraction is also necessary for the maximal formation.

Furthermore, a comparison of the activity of peroxide formation was made in various reciprocal combinations, such as tumor lipid × residue of normal liver, tumor residue × lipid of normal liver, normal lipid × normal residue or tumor lipid × tumor residue, with the object of finding out whether or not the properties of the lipid are altered in tumor tissues. It turned out that the peroxide value was greatly decreased when tumor lipid was added to the residue of normal liver, suggesting that some changes in the properties of the lipid are occurring

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in tumor tissues as a consequence of neoplasia and that these changes are responsible for the loss of the peroxide formation in tumor tissues.

Several experiments were also made on residue fractions with the aim of finding a clue to the mechanism of zinc activation in EDTA-treated homogenates. The results of these experiments were not very conclusive.

Material and Methods

Male adult albino rats and mice were used as material. Normal rat livers, rat primary hepatomas, rat fibrosarcomas (Umeda rhodamine sarcoma) and mouse Ehrlich ascites tumor respectively were examined. Mouse Ehrlich ascites tumor cells were separated from ascites serum by centrifuging at 800 r. p. m. for 10 minutes, and washed twice with a 0.9 per cent NaCl solution and then centrifuged at 500 r. p. m. for 10 minutes. The precipitated tumor cells were then suspended in small amounts of 0.9 per cent NaCl solution and centrifuged down at 12,000 r.p.m. for 10 minutes. The precipitate thus obtained consisted of packed tumor cells and an adequate amount of it was weighed as in the case of wet tissues.

Total lipid fractions were obtained by extracting tissues with a 1 : 1 mixture of ethanol-ether. Tissues weighed were homogenized in 15 volumes of 1 : 1 mixture of ethanol-ether and the homogenates were heated in boiling water bath for about 30 seconds, re-homogenized, and centrifuged. Then the end volume of supernatant was adjusted to the original volume of solvent (15 volumes). This supernatant was used in this experiment as the total lipid fraction. The residue remained after the removal of ethanol-ether extract of total lipid by centrifuging was washed with ethanol-ether, centrifuged, dried and re-homogenized in 1/15 M phosphate buffer solution of pH 5.9 equivalent to the amount of ethanol-ether for the total lipid extraction. These homogenates are described as "residue fraction" or "protein fraction" in the following. Further fractionation of the total lipid fraction was carried out by the method of Bloor (10). Each 10ml of total lipid fraction was evaporated on the boiling water bath, and the dried material was extracted three times with 6 ml of petroleum ether and filtered. The combined filtrate was concentrated on the boiling water bath to about 3 ml, and then 21 ml of acetone and 3 drops of $MgCl_2$-saturated ethanol were added. After 10 minutes standing, phospholipids were precipitated by centrifuging and the supernatant was discarded. The phospholipid fraction thus obtained was then suspended in 10 ml of 1/15 M phosphate buffer of pH 5.9. Choline-containing phospholipid and non-choline-containing phospholipid were separated by the method of Hevesy (11). 10 ml of ethanol-ether extract of the tissue (total lipid fraction) was evaporated and the dried material was extracted twice with 6 ml of petroleum ether. The

undissolved matter was removed by filtration. The petroleum ether extract was then evaporated and the dried material was dissolved in 30 ml of methanol. The methanol extract contains all phospholipids. Then 1.4 g of adsorptive magnesium oxide (Westvaco Chemical Division, U. S. A.) was added to 21 ml of this methanol extract, and after 30 minutes shaking, magnesium oxide was separated by centrifuging. By this procedure, non-choline-containing phospholipid was adsorpted to magnesium oxide and choline-containing phospholipid was left in the supernatant methanol solution.

One ml of ethanol-ether extract containing total lipid fraction was then evaporated and dried in a 50 ml vessel. To this dried material of total lipid fraction, 1/15 M phosphate buffer solution of pH 5.9 containing the substances to be tested such as $FeCl_3$, $ZnCl_2$, EDTA, residue equivalent to 66.6 mg of wet tissue, or dialyzed residue was respectively added as shown in the text. The end volume of the reaction mixture was always adjusted to 2 ml, and the end concentration of metals and EDTA was 0.001 M and 0.0001 M, respectively. In the case of phospholipid fraction, to 1 ml of phospholipid fraction suspended in a phosphate buffer, 1 ml of phosphate buffer, residue fraction equivalent to 66.6 mg of wet tissue, buffered $FeCl_3$, or buffered $ZnCl_2$ was added respectively. In the case of the methanol solution containing total phospholipid or choline-containing phospholipid, 3 ml of methanol solution was evaporated, dried, and 2 ml of residue fraction equivalent to 40 mg of wet tissue was then added.

For comparison, rat liver was homogenized in 15 volumes of 1/15 M phosphate buffer solution of pH 5.9, and the reaction mixture containing 1 ml of this homogenate plus 1 ml of buffer or buffered $FeCl_3$, $ZnCl_2$, and EDTA was also examined for the peroxide formation.

The reaction mixture thus obtained was then incubated aerobically at 37°C with shaking for 1 hour and at the end of incubation period, 2 ml of 20 per cent trichloroacetic acid and 4 ml of 0.67 per cent TBA were added, and then the mixture was placed in a boiling water bath for 15 minutes. The pink color appeared in the course of boiling. After cooling and centrifuging, 2 ml of supernatant were diluted with 6 ml of deionized water, and the optical density of pink color of this diluted solution was measured in a Hitachi photometer with a No. 50 filter. In the case of choline-containing phospholipid, however, colorimetric determination was carried out without diluting the sample, and corrected against the optical density of residue fraction alone. The results are expressed as $(-\text{Log } T)\times 100$ in this paper.

Results

Experiments on Normal Rat Liver

1. Comparison of lipid peroxide formation in lipid and residue fractions.

In the previous paper (9) it has been shown that the capability of lipid peroxide formation is nearly all lost when EDTA is added to the homogenate of these tissues and that it is restored to the normal level on addition of zinc. Iron has also been found to accelerate the formation of lipid peroxide when added to intact homogenates, although it is inactive in EDTA-treated homogenates.

In the present study, an attempt was first made to fractionate normal rat liver into two parts, *i. e.*, lipid fraction and residue (protein) fraction, and to examine for lipid peroxide formation in both the fraction. For the sake of comparison, experiments on whole homogenates were also repeated. Rat liver was homogenized in 15 volumes of 1/15M phosphate buffer solution and the TBA values were respectively measured as described above in the following systems : intact homogenates, homogenates plus Zn, homogenates plus Fe, EDTA-treated homogenates, and EDTA-treated homegenates plus Zn or Fe. As shown in Table 1, it was

Table 1. TBA values ((−Log T)×100) in whole homogenate, lipid fraction and residue fraction.

	No. 1	No. 2	No. 3	Average
Homogenate	23.7	20.0	24.8	22.8
Homogenate+Fe	28.8	24.2	30.3	27.8
Homogenate+Zn	21.5	13.0	18.4	17.6
Homogenate+EDTA	1.1	1.5	1.0	1.2
Homogenate+EDTA+Fe	6.0	7.7	15.4	9.7
Homogenate+EDTA+Zn	22.0	14.0	18.6	18.3
Lipid	2.0	1.4	0.5	1.3
Lipid+Fe	15.3	12.5	16.1	14.6
Lipid+Zn	2.4	1.4	1.0	1.6
Residue	2.6	1.8	1.9	2.1
Residue+Fe	3.3	2.0	2.3	2.5
Residue+Zn	2.7	1.6	1.4	1.9
Lipid+residue	22.5	21.7	27.0	23.7
Lipid+residue+Fe	25.0	23.0	33.3	27.1
Lipid+residue+Zn	17.2	15.0	22.3	18.2

again found that EDTA inhibited almost completely the formation of lipid peroxide and that zinc alone was effective in restoring this activity. Iron was also effective when added to intact homgenates as reported previously.

Next, a lipid fraction and the residue remaining after ethanol-ether extraction were obtained from the same homogenate and examined for lipid peroxide formation. It was found that neither the lipid fraction (TBA value= 1.3) nor the residue (2.1) alone showed appreciable TBA values. It was very striking to observe, however, that when the residue was added together to

the lipid fraction, the capacity of lipid peroxide formation was again restored to the normal level of whole homgenate (23.7). These results are given in Table 1. In this Table are also shown results of the addition of zinc or iron to these fractions. It is to be noted that the lipid fraction could respond to the addition of iron, whereas the residue showed little change. Zinc had little effect on either fraction. After the combination of both fractions, iron accelerated and zinc slightly retarded the formation of peroxide, as in the case of whole homogenates.

Having confirmed that zinc has little effect on the formation of peroxide in the lipid fraction alone whereas iron can accelerate the rate of formation, effects of these metals were tested on EDTA-treated lipid fractions. As shown in Table 2,

Table 2. TBA values in EDTA-treated lipid fraction.

	No. 1	No. 2	No. 3	Average
Homogenate	22.1	23.7	27.1	26.0
Lipid+EDTA	0.4	0.8	0.5	0.6
Lipid+EDTA+Fe	18.0	20.8	21.3	20.0
Lipid+EDTA+Zn	1.6	1.2	1.7	1.5

the TBA values were 26.0 in whole homegenates, 0.6 in EDTA-treated lipid fraction, 1.5 in EDTA-treated lipid plus zinc, and 20.0 in EDTA-treated lipid plus iron. Thus, zinc could not restore the peroxide formation in the lipid fraction to the homogenate level even when EDTA was added. However, in the case of lipid fraction, iron was again effective in promoting the peroxide formation after the treatment with EDTA. It appears therefore that although zinc is essential for the formation of lipid peroxide, it is acting by cooperating with some other cofactor presumably contained in the residue fraction.

To sum up, the results hitherto described have shown that both lipid and residue (protein) fraction are necessary for the formation of lipid peroxide and that zinc is effective only in whole homogenates treated with EDTA, being with-

Table 3. Effect of the ratio lipid to residue on the peroxide formation.

Lipid : residue	No. 1	No. 2	No. 3	Average
Lipid 2 : residue 1	19.8	26.5	21.8	22.7
Lipid 1 : residue 1	19.6	23.7	19.5	20.9
Lipid $\frac{1}{2}$: residue 1	18.3	17.5	16.5	17.4
Lipid $\frac{1}{4}$: residue 1	17.1	14.6	14.0	15.2
Lipid 1 : residue 2	24.3	31.4	30.0	28.6
Lipid 1 : residue 1	19.6	23.7	19.5	20.9
Lipid 1 : residue $\frac{1}{2}$	11.8	16.0	13.0	13.6
Lipid 1 : residue $\frac{1}{4}$	6.9	9.8	7.6	8.1

out effect in lipid fractions even when EDTA is present.

2. Comparison of lipid peroxide formation in systems in which the lipid fraction and the residue are combined in varying proportions.

In the previous section it has been shown that the addition of the residue fraction is necessary for the lipid fraction to produce lipid peroxide in amount nearly equal to the level of original homogenates.

The capability of peroxide formation was then studied in systems in which the lipid fraction and the residue were combined in varying ratios. The original lipid fraction and the residue fraction contains respectively the materials equivalent to 66.6 mg of wet liver and were mixed in varying proportions.

Results of the experiments are shown in Table 3 and Figure 1. It will be seen that the maximal peroxide formation occurs when the ratio lipid to residue is 2 : 1, as well as when the ratio residue to lipid is 2 : 1, indicating that a sufficient amount of both fractions is needed. However, it is to be noted that peroxide value is increased proportional to the amount of residue fraction added, when the lipid content is constant. This may suggest that residue is also of importance in the production of lipid peroxide.

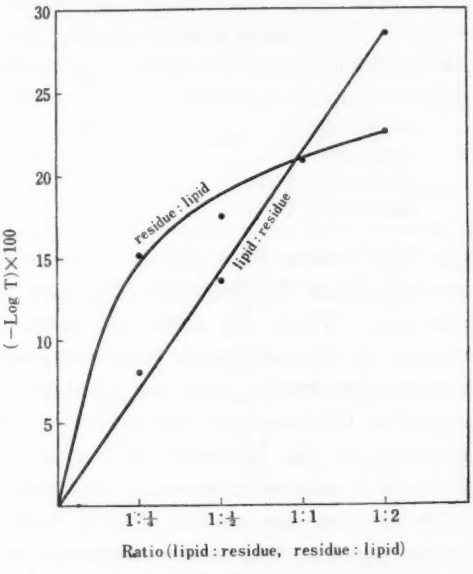

Fig. 1 Effect of the ratio, lipid : residue and residue : lipid, on the peroxide formation.

3. Effect of dialysis of the residue on the peroxide formation in the lipid-residue system.

In order to examine whether or not a cofactor of peroxide formation is involved in the residue fraction, experiments were performed on systems lipid plus residue, but using this time dialyzed residue. 1.67 g of liver was extracted in 25 ml of ethanol-ether and the residue was washed, dried, and again homogenized in 25 ml of 1/15 M phosphate buffer solution. Then 20 ml of this homogenate were put in a cellophane bag and dialyzed against 2 *l* of deionized water in a refrigerator. 5 ml of homogenate of undialyzed residue and the lipid extract were also placed in the same refrigerator. After 48 hours 4 samples of 1 ml of lipid extract were evaporated and dried, and these dried samples were examined for

peroxide formation by adding the following solutions respectively: (1) 1 ml of homogenate of the untreated residue plus 1 ml of buffer solution, (2) 1 ml of dialyzed residue plus 1 ml of buffer solution, (3) 1 ml of dialyzed residue, 0.5 ml of buffered solution of $FeCl_3$ (1/250 M) and 0.5 ml of the buffer solution, and (4) 1 ml of dialyzed residue, 0.5 ml of buffered solution of $ZnCl_2$ (1/250 M) and 0.5 ml of the buffer solution. The results are shown in Table 4.

Table 4. Effect of dialysis of the residue on the peroxide formation.

	No. 1	No. 2	No. 3	Average
Lipid+residue	13.3	8.2	19.1	13.5
Lipid+dialyzed residue	1.8	2.9	2.2	2.3
Lipid+dialyzed residue+Fe	9.9	5.7	10.4	8.7
Lipid+dialyzed residue+Zn	1.1	1.5	1.9	1.5

It will be seen that whereas a considerable amount of lipid peroxide is formed in the system lipid plus undialyzed residue, its formation is greatly reduced in the system lipid plus dialyzed residue, indicating that a dialyzable cofactor contained in the residue fraction is essential for the formatien of the peroxide. Zinc does not appear to act as substitute for this dialyzable cofactor. A promoting effect of iron observed may also be explained as due to its action on lipid fraction (cf. Table 1). Thus, it seems very difficult to explain for the present how the restoration of reduced activity by EDTA is effected by zinc ions in the whole homogenate. However, it appears that zinc is the primary activator involved in the natural state, for iron can produce a further promoting effect only after zinc activation has occurred in EDTA-treated homogenates (unpublished results).

4. Further fractionation of the lipid fraction.

Now the question may be raised as to what kind of lipid is involved in the process of this peroxide formation. Therefore, the lipid fraction of the liver was further fractionated by the method of Bloor (10), and it was found that phospholipid was the only fraction capable of forming peroxide, when the residue

Tabie 5. TBA values in phospholipid fraction.

	No. 1	No. 2	No. 3	Average
Lipid	1.1	1.7	1.1	1.3
Lipid+residue	20.4	29.0	21.0	23.5
Phospholipid	6.2	7.2	5.3	6.2
Phospholipid+residue	30.2	35.6	29.8	31.9
Prospholipid+Fe	17.0	18.2	18.2	17.8
Phospholipid+Zn	6.9	6.0	5.7	6.2

was added. Furthermore, it was found that among the phospholipids non-choline-containing phospholipid was responsible for the formation of this peroxide, when fractionation was done according to the method of Hevesy (11), using MgO in methanol extract.

In Table 5 are given the results of experiments in which the activity of the

Table 6. TBA values in total phospholipid, choline-containing phospholipid and non-choline-containing phospholipid.

Tissues	Total phospholipid	(a) Choline phospholipid	(b) Non-choline phospholipid*	(a) : (b)
Rat liver	49.0	1.3	47.7	3 : 97
Rat brain	29.8	8.7	21.1	29 : 71
Rat kidney	8.0	1.6	6.4	20 : 80
Rat heart muscle	5.7	1.2	4.5	21 : 79

* These values are the difference between total phospholipid and choline phospholipid values.

peroxide formation was compared in phospholipid and total lipid fractions. It is obvious that phospholipid fraction is more active than total lipid fraction. It is also to be noted that iron has an accelerating effect when added to this phospholipid fraction.

That among the phospholipids a non-choline-containing phospholipid fraction is much more effective in producing peroxide than choline-containing phospholipid, is demonstrated in Table 6.

Experiments on Lipid Peroxide Formation in Tumor Tissues

1. Reciprocal combinations tumor fractions × normal fractions.

In the previous paper (9), it has already been shown that the activity of lipid peroxide formation is considerably decreased in livers of tumor-bearing animal and that tumor tissues lose much of this activity. In the present study, therefore, experiments were performed using lipid and residue fractions obtained from various tumor tissues together with reciprocal combination experiments such as tumor lipid × normal residue or tumor residue × normal lipid. Such a comparison of the activity of peroxide formation in various reciprocal combinations (tumor × normal) was expected to reveal the chemical changes which are responsible for the loss of this activity in tumor tissues.

Lipid fractions and residue fractions employed in these experiments were respectively equivalent to 66.6 mg of wet tissue whether in normal or tumor tissues, and a 1 : 1 mixture of both fractions was contained in 2 ml of incubation medium.

First, the activity of peroxide formation was compared using fractions obtained from rat fibrosarcomas and normal rat liver. Reciprocal combinations examined were as follows: normal lipid × normal residue, normal lipid × sarcoma residue, sarcoma lipid × normal residue, and sarcoma lipid × sarcoma residue. Results are represented in Table 7. It will be seen that the TBA value is 15.6 on an

Table 7. TBA values in reciprocal combination of fibrosarcoma fractions × normal liver fractions.

No.	Normal liver lipid / Fibrosarcoma lipid × Normal liver residue / Fibrosarcoma residue			
	Normal lipid × Normal residue	Normal lipid × Sarcoma residue	Sarcoma lipid × Normal residue	Sarcoma lipid × Sarcoma residue
1	21.2	26.2	1.4	0.8
2	16.8	24.6	10.8	1.3
3	15.0	17.8	2.6	0.9
4	14.4	19.3	8.3	6.6
5	13.0	22.5	5.5	1.0
6	13.0	26.2	3.7	0.5
Average	15.6	22.8	5.4	1.9

average in the case of normal liver lipid plus normal liver residue, and 1.9 in sarcoma lipid plus sarcoma residue, indicating again that tumor tissue has lost much of its activity with regard to the peroxide formation. It is also to be seen that sarcoma residue is effective in forming the peroxide, when normal lipid is added. However, it is extremely interesting to observe that the peroxide

Table 8. TBA values in reciprocal combination of hepatoma fractions × normal liver fractions.

No.	Normal iiver lipid / Hepatoma lipid × Normal liver residue / Hepatoma rasidue			
	Normal lipid × Normal residue	Normal lipid × Hepatoma residue	Hepatoma lipid × Normal residue	Hepatoma lipid × Hepatoma residue
1	15.7	1.4	1.4	1.4
2	17.0	11.2	1.7	0.8
3	16.6	14.3	9.5	1.5
4	17.7	7.5	12.5	6.6
5	9.5	1.6	4.3	1.1
6	17.2	2.2	2.0	0.8
Average	15.6	6.4	5.2	2.0

value is greatly decreased when sarcoma lipid is added to normal residue. This may suggest that certain changes in the properties of the lipid as a consequence of neoplasia are responsible for the loss of the peroxide formation in tumor tissues.

Experiments were then made using primary rat hepatomas and normal rat livers, and similar results were obtained (Table 8). The activity of peroxide formation was greatest in normal lipid × normal residue and least in hepatoma lipid × hepatoma residue, and the activity was less in hepatoma lipid × normal residue than in normal lipid × hepatoma residue. In the case of hepatoma, however, tumor residue was not so effective as in the case of fibrosarcoma, when added to normal lipid. At any rate, there was a definite decrease in the peroxide formation, when hepatoma lipid was added to normal residue.

Furthermore, similar experiments were done on combined systems Ehrlich ascites tumor fractions × normal liver fractions. As shown in Table 9, similar

Table 9. TBA values in reciprocal combination of Ehrlich ascites tumor fractions × normal liver fractions.

No.	Normal liver lipid ⤫ Ehrlich ascites tumor lipid; Normal liver residue ⤫ Ehrlich ascites tumor residue			
	Normal lipid × Normal residue	Normal lipid × Ascites tumor residue	Ascites tumor lipid × Normal residue	Ascites tumor lipid × Ascites tumor residue
1	17.6	6.8	4.0	2.3
2	22.8	9.3	3.4	2.1
3	16.5	8.4	3.4	1.8
4	20.8	10.7	2.3	1.6
5	20.0	7.4	2.7	2.3
Average	19.5	8.5	3.2	2.0

results were obtained. As in the case of hepatoma, the residue fraction of the ascites tumor could not regain the normal level of TBA value, even when added to normal lipid. However, the decrease in TBA values was more marked when tumor lipid was combined with normal residue.

From these results it may be concluded that the property of lipid (presumably phospholipid) have undergone a qualitative change in tumor tissues and as a result the peroxide formation no longer occurs.

2. Absence of antioxidants from the residue fraction of tumor tissues.

However, the possibility that residue (protein) fraction is also qualitatively altered in tumor tissues is not excluded, since no appreciable increase in the lipid peroxide was observed when the residue of hepatoma or ascites tumor was

added to normal lipid fraction. It seemed that the residue of these tumor tissues contain some antioxidant. Therefore, it was decided to examine whether the peroxide values in normal lipid × normal residue are influenced by further addition of tumor residue. A considerable decrease will be found if the latter contains antioxidants. 2 ml of incubation mixture containing lipid and residue fractions of normal rat liver (equivalent to 66.6 mg of wet tissue, respectively) plus tumor residue (equivalent to 33.3 mg or 66.6 mg of wet tissue) were incubated and the TBA value was measuered. As shown in Table 10, the residue of these

Table 10. Effect of the tumor residue on the lipid peroxide formation in normal liver.

	No. 1	No. 2	No. 3	Average
Rat liver lipid+rat liver residue	22.8	16.5	20.8	20.0
Rat liver lipid+rat liver residue+ Ehrlich ascites tumor residue	27.0	22.6	23.5	24.4
Rat liver lipid+rat liver residue+$\frac{1}{2}$vol. Ehrlich ascites tumor residue	25.6	21.5	23.2	23.4
Rat liver lipid+rat liver residue	17.2			
Rat liver lipid+rat liver residue+rat hepatoma residue	20.9			
Rat liver lipid+rat liver residue+$\frac{1}{2}$vol. rat hepatoma residue	20.7			

tumor tissues had little effect on the peroxide formation occurring in the system normal lipid × normal residue. Thus, it does not appear that tumor residue has an appreciable amount of antioxidant.

Discussion

It has been demonstrated in the present study that the activity of lipid peroxide formation is hardly detectable in three kinds of tumor tissues whether in whole homogenate or its fractions, whereas in normal tissues the peroxide is formed in appreciable amounts on aerobic incubation. The first problem is whether or not the lack of this activity in tumor tissues is due to a low concentration of lipid in these tissues. However, Yasuda and Bloor (12), Bierich and Lang (13), and Pentimalli and Schmidt (14) have reported that malignant tumors show an increase in phospholipid content in direct proportion to the degree of malignancy, although Kishi, Fujiwara and Nakahara (15) reported a slight decrease in phospholipid content of hepatoma as compared with that of normal liver.

Secondly, it may be questioned whether or not tumor phospholipid shows a lower iodine number than the lipid of normal tissues. According to Yasuda (16)

the iodine number of the tumor phospholipid is lower than that of liver, and Haven (17) also reported that the degree of unsaturation of the phospholipids is lower in tumor than in muscle or liver. Thus Haven and Bloor (18) suggested that low iodine numbers such as found by Yasuda for unsaponified phospholipid, and by Haven for phospholipid fatty acids are due to the presence of a comparatively saturated phospholipid in the tumor. Shuster (6), however, indicated that the concentration of linolenic acid is higher in the ascites tumor than in rat liver, although the TBA test is negative in the tumor and positive in normal liver. Thus, Shuster has suggested that there is present in tumor tissues some antioxidant which is capable of inhibiting the peroxide formation. Wolfson, Wilbur and Bernheim (7) have also suggested an increased antioxidant activity in regenerating liver, and Bernheim, Ottolenghi and Wilbur (8) indicated that the formation of peroxide in liver homogenates is abolished when the suspension of bone marrow is added, suggesting the presence of antioxidants in the latter. However in the present study, it has definitely been shown that rat fibrosarcomas, hepatomas and Ehrlich ascites tumor contain no antioxidant whatever.

Therefore, there seems to be considerable difficulty in deciding how the inhibition of peroxide formation is brought about in tumor tissues. However, evidence has been described in the present paper that at least in the case of normal tissues, their phospholipid fraction, especially the non-choline phospholipid fraction prepared by the method of Hevesy (11), is very effective in producing peroxide and that when tumor lipid is added together to normal residue, the peroxide forming activity is almost completely abolished. This may suggest that in tumor tissues a certain kind of phospholipid is qualitatively altered in the direction of saturation or rapidly decreasing in its content. In other words, the general impression is that tumor tissues are different from normal tissues in the nature of phospholipid constituents, and that the modification or the lack of certain phospholipid constitutes an important characterization of the tumor tissue. The nature of this phospholipid which is responsible for lipid peroxide formation is still unknown, but the author is now obtaining some evidence that it is a certain kind of acetal phosphatide (plasmalogen).

The third problem to be discussed is whether or not the residue (proten) fraction is also altered as a tumor develops. It has been found that in the case of hepatomas and Ehrlich ascites tumor, the residue fractions of these tissues exert a marked inhibiting influence when added to the lipid fraction of the normal liver, although in the case of fibrosarcomas, their residue fraction produces a slight accelerating effect upon the normal lipid. Thus, it seems that some changes are also occurring in the properties of residue in tumor tissues.

However, it is to be noted that the changes in the activity with respect to the peroxide formation are more pronounced in the lipid fraction than in residue.

Further, it has been shown that if the residue is dialyzed, its peroxide forming activity is almost completely abolished, even on addition of the lipid fraction. Therefore, a cofactor of peroxide formation which is contained in the residue and is readily dialyzable, seems to play an important part in this reaction.

Lastly, the relationship between the peroxide forming activity and the role of metals will be discussed. First, zinc has been found to have a marked enhancing effect when added to EDTA-inactivated whole homogenates, although it has no effect on intact homogenates or its lipid or residue fraction alone. It is also without effect on EDTA-treated lipid fractions. It is therefore very difficult to explain how the activation by zinc is effected in the case of EDTA-treated homogenates. It appears, however, that unlike the case of iron, zinc is a natural activator involved in the original homogenate and is acting in cooperation with a cofactor contained in the residue fraction. It may also be assumed that in tumor tissues, zinc is not freely available for the reaction, for there is some evidence that zinc in hepatoma is hardly freed or extracted from its firmly bound state as compared with the case of normal tissues (19).

On the other hand, iron has been shown to have an accelerating effect whether in intact whole homogenates or lipid fractions, in contrast to the case of zinc. This effect is quite naturally expected, since it is generally known that iron has a catalytic action on the autoxidation of unsaturated fatty acids (20, 21, 22, 23). However, it is to be noted that normal liver residue which must contain a sufficient amount of iron in such forms as hematin is not able to regain the peroxide forming activity, when added to tumor lipid.

Summing up, it seems that some qualitative changes in phospholipid occur and at the same time zinc becomes no longer freely available during the course of tumor development, these changes exerting an overall inhibition of the lipid peroxide formation in tumor tissues. It may also be stated in general that for the formation of peroxide to occur a phospholipid and a cofactor in the residue are essential together with zinc. As regards the properties of phospholipid responsible for peroxide formation further studies are now being carried on.

Summary

1. Homogenates of normal and tumor tissues were respectively fractionated into lipid and residue (protein) fractions and these fractions were examined for lipid peroxide forming activity. It was found that whereas neither the lipid nor residue fraction alone showed apperciable activity, the combination of both fractions invariably showed a high peroxide value, at least in the case of normal tissues.

2. Lipid which is responsible for the peroxide formation was found to be phospholipid.

3. When the residue fraction was dialyzed, the peroxide forming activity was no longer observed even after the addition of an active lipid fraction, suggesting the persence of a dialyzable cofactor in the residue. It was therefore concluded that for the production of lipid peroxide a phospholipid and a cofactor are essential together with zinc.

4. Iron alone could activate the lipid peroxide formation in whole homogenates or lipid fractions, although zinc has been found to be the only metal which can restore the activity in EDTA-treated homogenates.

5. A comparison of the activity of peroxide formation was made in various reciprocal combinations (tumor fractions × fractions of normal liver). Having confirmed that capability of peroxide formation is nearly all lost in tumor tissues, it was further found that the peroxide value was greatly decreased when tumor lipid was added to the residue of normal liver.

6. Reduced activity in tumor tissues with respect to peroxide formation is not due to the presence of antioxidants.

7. Suggestions were put forward that as the result of neoplasia some qualitative changes in phospholipid occur and at the same time zinc becomes firmly bound to cell structures and no longer available for the reaction, these changes leading to an overall inhibition of the lipid peroxide formation in tumor tissues.

8. The general impression was obtained that the tumor tissue is different from normal in the nature of phospholipid constituents, and that the modification or the lack of certain phospholipids constitute an important characterization of the tumor tissue.

Acknowledgement

The author wishes to express his gratitude to Professor T. Fujii for his guidance and encouragement. For the specimens of Ehrlich ascites tumor and of fibrosarcoma used, he wishes to thank Professor Y. Kimura, University of Tokyo, and Dr. W. Nakahara, Director of Cancer Institute, Tokyo.

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要　旨

癌組織における lipid peroxide の形成と燐脂質

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1. 白ネズミの正常組織を脂質分劃と，脂質を抽出したあとの残渣分劃との二つにわけ，そのおのおのについて lipid peroxide の形成をしらべた。これらの分劃は単独では lipid peroxide を形成しないが，二つをくみあわせると lipid peroxide の形成がみられる。

2. 脂質をさらにいろいろの分劃にわけて peroxide の形成をしらべたところ，燐脂質が peroxide をつくることがわかった。

3. 透析した残渣分劃と脂質分劃とのくみあわせでは lipid peroxide の形成がみられないから，lipid peroxide の形成には透析性の補助因子が必要である。

4. 鉄は単独で，組織または脂質分劃にはたらいて lipid peroxide の形成を促進するが，亜鉛は EDTA で処理した組織の場合だけに効力がある。したがって正常組織における lipid peroxide の形成には，燐脂質のほかに，透析性の補助因子と亜鉛とが必要である。

5. 癌組織の脂質分劃に正常組織の残渣分劃を加えても lipid peroxide が形成されない。

6. 癌組織で lipid peroxide の形成のみられないのは，癌組織に antioxidant があるからではない。

7. 癌組織では燐脂質が変化し，そのうえ細胞構造と亜鉛との結合がきわめて強いために lipid peroxide の形成がみられないものとおもわれる。

8. 癌組織では，ある種の燐脂質が完全に飽和化されて質的にかわってしまっているか，量的に激減しているかのいずれかであると考えられる。

(文部省科学研究費による)

[GANN, 49, 249~259; December, 1958]

PART I. POLAROGRAPHICAL, ELECTROPHORETICAL AND BIOCHEMICAL STUDIES ON THE HIGH-MOLECULAR CONSTITUENTS OF GASTRIC JUICE WITH REFERENCE TO CANCER OF THE STOMACH

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In spite of several patho-histological studies presented on "pre-cancerous stage of the stomach", little has been known on the changes of gastric secretion, especially of its high-molecular constituents, in this pathological condition.

With a view to difining a "pre-cancerous stage of the stomach" clinically, an attempt has been made to find, by means of polarography, electrophoresis and biochemical analyses, a change in the secretion of high-molecular constituents of gastric juice which is accompanied by several chronic pathological conditions, especially cancer.

Materials and Methods

Studies were carried out on subjects with gastric cancer, and with other chronic pathological conditions of the stomach (chronic gastritis and peptic ulcer, with hypo- or anacidic response to insulin stimulation)[1)2)] and on 10 cases each of healthy controls of both sexes.

After fasting for about 12 hours, aspiration of gastric juice was made through a small tubing, followed by an intravenous injection of 10 units of insulin and subsequent aspirations of gastric juice at intervals of 20 minutes for 1 hour.

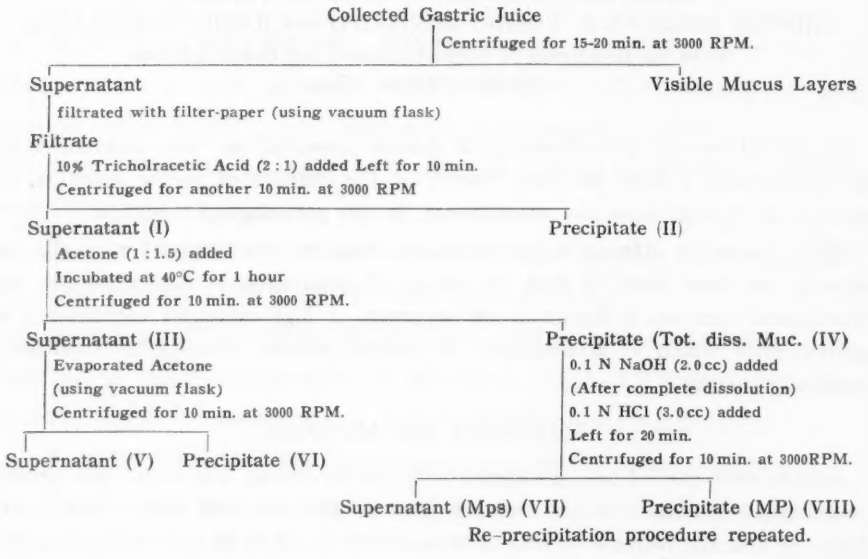
During aspiration of the specimens, the greatest possible care was taken to avoid contaminations with saliva, blood and bile.

The specimens were subsequently filtered through a layer of cotton wool to remove visible mucus and other contaminants. The filtrate was submitted immediately to polarographical, electrophoretical and several chemical examinations.

Each of the samples of gastric juice devided into three groups, namely gastric cancer group, anacidic group with chronic pathological conditions, and acidic controls, was adjusted to approximately pH 1.0, 4.0, 7.0, and 11.0 by adding 1 *n* sodium hydroxide or 1 *n* hydrochloric acid. The polarographic filtrate wave[3)] was measured on each of the samples.

Filtrate specimens were then devided into 2 groups, namely, the cancerous specimens and the noncancerous specimens (controls), and were fractionated according to the method of Glass and Boyd,[4] as is seen from Tab. 1. On each of the fractions thus obtained, polarographic and electrophoretic behaviours were followed up and a series of chemical analyses was made simultaneously. The "filtrate method" of Waldschmidt-Leitz for serum[3] was adopted for the polarography of gastric juice.

Table 1. Method for Fractionation of Gastric Juice



Electrophoresis was carried out as follows: Each specimen, introduced into a cellophane sack, was dialysed and condensed simultaneously on Carbowax 1500 at 0°–5°C, the protein concentration was brought to approximately 3 per cent. The paper electrophoresis was carried out with these specimens in both veronal buffer (pH 8.6, μ=0.1, 10 V/cm) and acetate buffer (pH 4.5, μ=0.1, 10 V/cm).

The following methods were used for chemical determinations: Töpfer's standard titration for the acidity, Kjeldahlometry for nitrogen, methods of Glass and Boyd for tyrosine[4], of Elson-Morgan-Shida[5][6] for hexosamine (as glucosamine) and of Ishidate and Nambara[7] for hexuronic acid (as glucuronic acid).

The diphenylamine[8] (referred to as DPA) reaction was performed in the following way: To each specimen in a Pyrex tube (25×150 mm) was added a half volume of 10% trichloracetic acid. The tube was gently shaken, and was covered with a glass stopper. The tube was then heated in a boiling water-bath for exactly 20 minutes. The tube was then removed from the bath and was cooled

by immersing in cold water. The specimen was filtered.

Three ml of 1% DPA reagent (90.0 ml of glacial acetic acid plus 10.0 ml of concentrated sulphuric acid plus 1 g of DPA) was added to 1.5 ml of the filtrate. The mixture was heated for exactly 30 minutes in a boiling water-bath and was then cooled immediately in running cold water. The optical density of purple colour developed was read in a spectro-photometer at 530 mμ against its blank containing the specimen, 10% trichloracetic acid, glacial acetic acid and concentrated sulphuric acid.

Results

A. Experiments with the fasting and insulinstimulated specimens of gastric juice.

(1) *Polarographic observations*:

a) Changes in the polarographic filtrate waves (Pfw) of gastric juice related to the adjustment of pH of the specimens.

The changes in Pfw caused by adjusting the pH were shown in Fig. 1. The most remarkable change was observed in the cancerous samples among the above three groups. A relatively marked change was observed in the anacidic sample, while the changes in the acidic control sample was the lowest. In other words, the descending order of the lability of Pfw is as follows: the sample of gastric cancer, of the other anacidic chronic pathological conditions and the control sample.

b) Comparison of Pfw of gastric juices before against after insulin-injection.

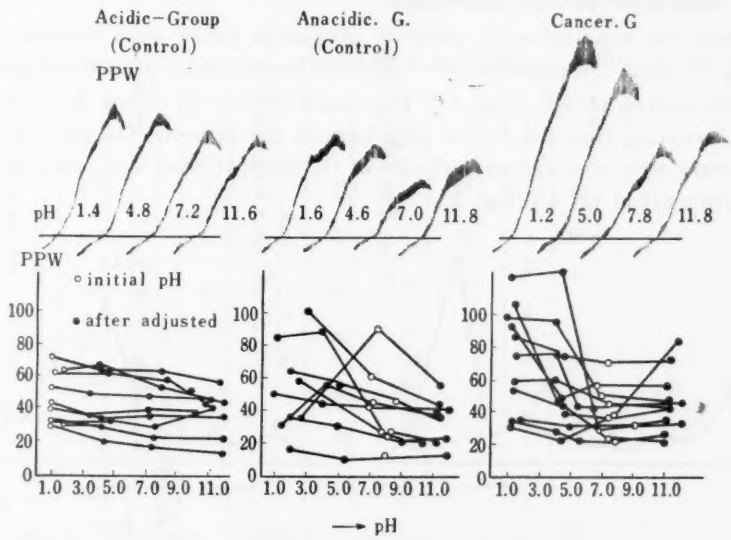


Fig. 1. Influence of pH-adjustment on the polarographic filtrate waves.

Comparing the Pfw of fasting gastric juice with those of 20 and 40 minute aspiration samples, a lowering of Pfw of 20 minute specimen was observed in the samples of cancer, as shown in Fig. 2, whereas in the other samples, a gradual rise was observed after the insulin-injection.

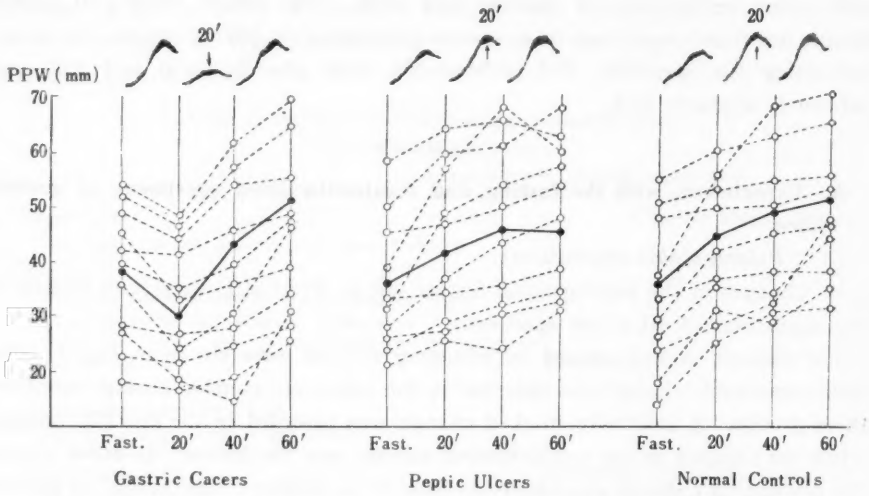


Fig. 2. Comparison of response to insulin injection in polarographic filtrate waves of gastric juices with healthy control, peptic ulcer, and with gastric carcinoma.

(2) *Paper-electrophoretic observations*:

Although the electrophoretic patterns of gastric juices were variable as was reported by many investigators,[9),10),11),12),13),14)] 5 main peaks were found generally in our pattern at pH 8.6, (Fig. 3). They were tentatively named B_1 to B_5 by the authors, counting from the fastest peak towards the slowest. On the other hand, 2 or 3 peaks were observed on each side of the original point when electrophoresis was carried out at pH 4.5 (Fig. 3).

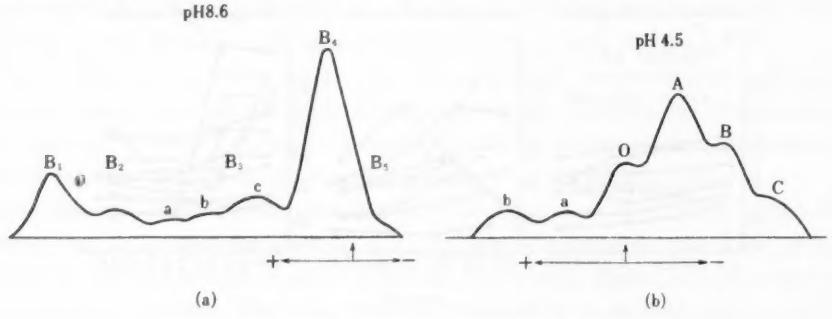


Fig. 3. Paper electrophoretic patterns of gastric juice.

Relatively large peaks coinciding with B_2 to B_4 peaks were noticed in the electrophoretic pattern of the cancerous specimen at pH 8.6, but no certain change was seen at pH 4.5.

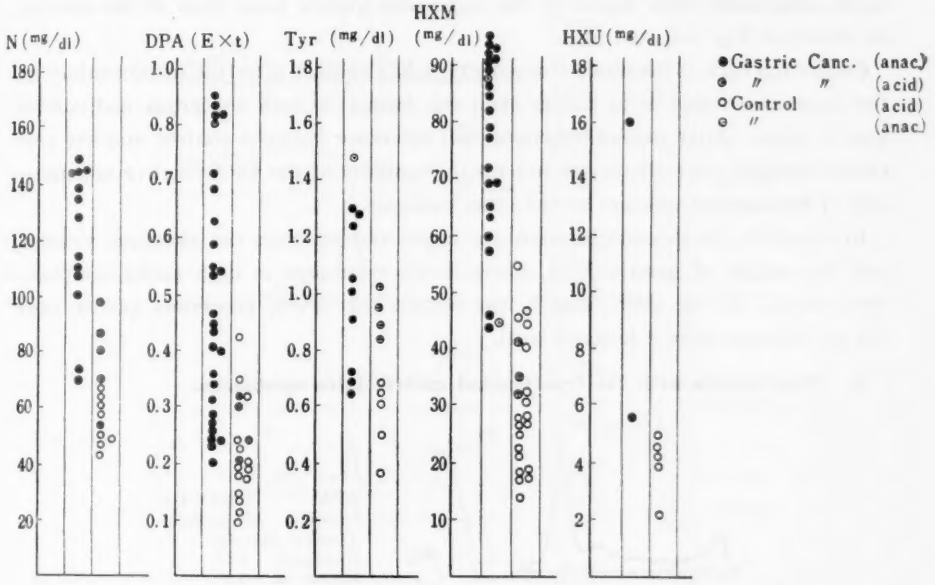


Fig. 4. (a) Several chemical determinations of gastric juices (Fasting)

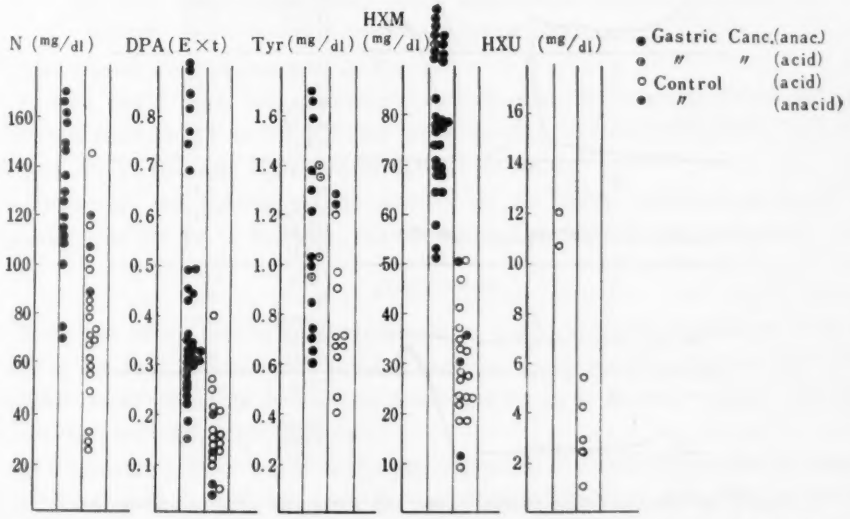


Fig. 4. (b) Several chemical determinations of gastric juice (after Insulin injection)

(3) *Several chemical determinations*:

The contents of nitrogen, tyrosine, hexosamine, hexuronic acid and of the DPA-reactive substance were determined on each gastric juice. All the contents of these components were higher in the cancerous gastric juice than in the control, as shown in Fig. 4-a, and -b.

Comparing each of the above components before against after the insulin-injection, the latter was found to be higher than the former in both concerous and control gastric juice. After insulin-injection, the difference between control and the concerous samples were distinctive in both the contents of the DPA-reactive substance and of hexosamine, but not in the other contents.

In regard to the relation between the above results from the chemical analysis and the acidity of gastric juice, lower levels were seen in high acidic sample of the control. On the other hand it was notable that acidic cancerous gastric juice did not always show a lowered level.

B. Experiments with the fractionated gestric juice specimens.

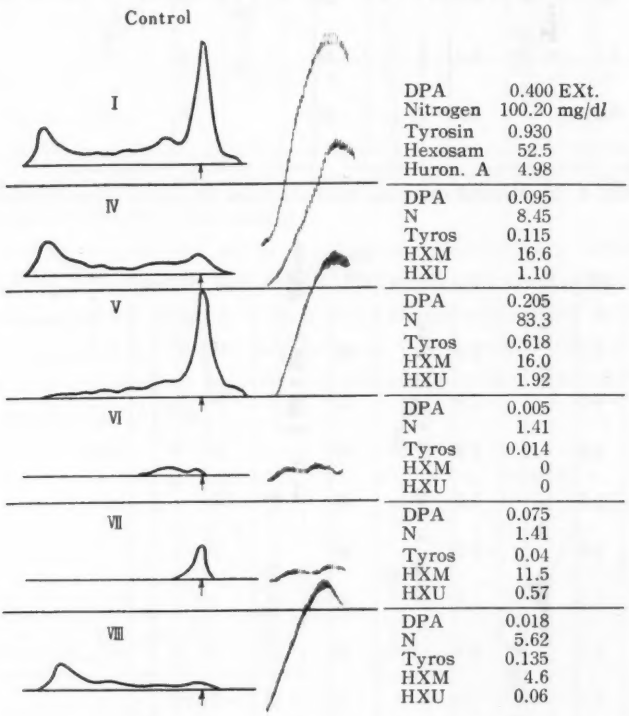
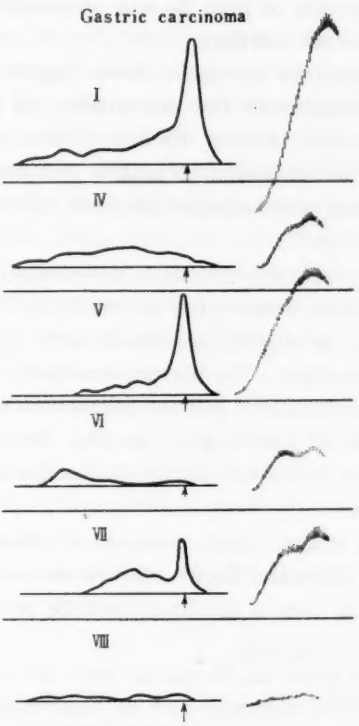


Fig. 5. (a) Polarographic filtrate waves, electrophoretic patterns and several chemical determinations of fractionated gastric juice.



DPA	0.742 EXt.
Nitrogen	192.2 mg/dl
Tyros	1.40
HXM	124.0
HXU	12.1
DPA	0.184
N	7.03
Tyros	0.161
HXM	37.0
HXU	2.92
DPA	0.348
N	16.02
Tyros	1.044
HXM	30.0
HXU	3.68
DPA	0.032
N	5.62
Tyros	0.06
HXM	0
HXU	0.30
DPA	0.168
N	18.27
Tyros	0.208
HXM	55.0
HXU	2.45
DPA	0.002
N	0
Tyros	0.023
HXM	0
HXU	0

Fig. 5. (b) Polarographic filtrate waves, electrophoretic patterns and several chemical determinations of fractionated gastric juice.

The results were summarized in Fig. 5.

It was found with the cancerous specimen that the polarographical activity removed from Fr. IV to Fr. VII, but little activity was detected in Fr. VIII. Fr. V and Fr. VI had also high polarographical activities.

Conversely, the polarographical activity of the acidic control was found to remove from Fr. IV to Fr. VIII, and the activity in Fr. VII was negligible.

Discussion

From the above results in polarographical study, cancerous specimen shows a lability of Pfw and a characteristic lowering in 20 minute aspirate, and also exhibits relatively large peaks corresponding with B_2 to B_4 and a single low peak B_1 in the electrophoretical pattern.

In comparative observations of the electrophoretical pattern with Pfw 20 minute aspirate, it may be said that peak B_1 was mainly connected with Pfw, since the increase of peak B_1 ran in parallel with the rise of Pfw in the control gastric

juice, and it was noted that the decrease of peak B_1 was sometimes accompanied by the lowering of Pfw in the cancerous sample.

In the chemical assays, the cancerous specimen shows higher contents of nitrogen, tyrosine, hexuronic acid, hexosamine and particularty the DPA reactive substance as compared with the control. Among the above chemical assays, the DPA reaction is the most useful for diagnosis of gastric carcinoma, since the optical density in 40 minute samples of carcinomatous specimen is distinctly higher than that of noncarcinomatous specimen.

As the result of statistical inquiry of analytical data, the nitrogen content was found to be correlated with the tyrosine content but not with the other contents. The tyrosine content was found to be slightly correlated with the contents of DPA-reactive substance and of hexosamine. The highest correlation was observed between the content of DPA-reactive substance and the hexosamine content.

In experiments with the fractionated gastric juice samples, the electrophoretic pattern of Fr. I consisting of 5 main peaks was similar to the pattern of gastric juices before submitting to filtration.

Fr. IV containing total dissolved mucin, which consisted of mucoproteose (Ms) and mucoprotein (Mp) according to Glass and Boyd[4], showed electrophoretically 2 well-defined peaks (B_1 and B_4) in the control specimen, and B_1 peak removed to Fr. VIII and B_4 to Fr. VII.

Contrary to the control, ill-defined peaks (B_2, B_3 and B_4) were seen in the electrophoretic pattern of cancerous specimen, a greater part of these peaks characteristically removing to Fr. VII, whereas hardly any peak being observed in Fr. VIII.

The fact that Fr. VII, which is the supernatant from Fr. IV, consisted electrophoretically of a single peak (B_4) in the control specimen, suggests a possible connection of this fraction with Ms. In the control, this fraction displayed no polarographic activity and the levels of the content of substances analysed chemically were low.

Conversely, the electrophoretical pattern of the cancerous specimen showed the ill-defined peaks (B_2, B_3 and B_4) and the polarographical activity was found to exist coinciding with relatively high levels of the DPA reaction and the hexosamine content. The Fr. VII in the cancerous specimen, therefore, might involve the mixed pathological products.

Fr. VIII, which consisted electrophoretically of a single peak (B_1), possibly has a certain relation to Mp. It was also noticed that the B_1 peak in the electrophoretic pattern of the control specimen was accompanied with the rising level of Pfw, and had relatively higher contents of nitrogen, tyrosine, and hexosamine, contrary to the observation that in the cancerous specimen B_1 peak was very small, and consisted of a negligible Pfw and the lower levels of contents of the

other substances.

Thus, it may be said that in gastric cancer, activity of Pfw and the content of substances mentioned above are relatively high in Fr. VII, which consisted mainly of B_2, B_3 and B_4 peaks in the electrophoretic pattern. In the control, contrary to the finding, a relatively high polarographic activity was observed in Fr. VIII, which consisted of a single electrophoretical peak B_1.

Furthermore, Fr. V is a fraction which is soluble in trichloracetic acid and in acetone. All the peaks with the exception of B_1 were observed in this fraction electrophoretically. In this respect, Fr. V is similar to Fr. I. The Pfw in both the control and the cancerous specimens were high and showed a perpendicular figure similar to that of native gastric juice.

The lability of Pfw in the native gastric juice may possibly be originated by the characteristics of this fraction. With exceptions of the contents of the DPA-reactive substance and of hexosamine, the contents of substances determined chemically were also high in this fraction.

In Fr. VI, few characteristics were observed both in the cancerous and in the control specimens.

Simultaneous examinations of the anacidic specimens in chronic pathological conditions are in progress.

Summary

1) Cancerous gastric juices were more labile polarographically than those of the anacidic and control samples.

2) Comparing the Pfw of each specimen of gastric juice aspirated at intervals of 20 minutes after insulin-injection, a transient lowering of Pfw of a specimen taken at the first 20 minutes was notable in gastric cancer.

3) From the chemical determination, the content of nitrogen, tyrosine, DPA-reactive substance, hexosamine and hexuronic acid was found to be in the descending order: Cancerous, anacidic, and acidic (control) samples.

4) As the result of statistical inquiry of the analytical data, positive correlations were found to exist between the DPA-reactive substance and the hexosamine content. No correlation was noticed between the combined content of tyrosine-nitrogen and that of DPA-hexosamine, so that the substances increased in cancerous gastric juice may be devided into two different groups, i. e. the nitrogen-tyrosine group and the DPA-hexosamine group.

5) From the result of acidic control, a physiologically active substance in Pfw (typical double wave) was noticed in Fr. VIII, which consisted electrophoretically of a single peak B_1, and into which the grandular Mp has been fractionated by Glass and Boyd.

6) Contrary to the above finding, the polarographically active substances in the cancerous specimens were observed in Fr. VI and VII, which consisted electrophoretically of the ill-difined peaks, B_2, B_3 and B_4. The increases in the content of DPA-reactive substance and of hexosamine seemed to be related to these peaks appeared in Fr. VI and Fr. VII.

7) The anacidic specimen was placed between the acidic control and the cancerous specimen in view of the lability of Pfw and the characteristics of chemical composition, but it resembled, in the nature, more closely to the cancerous specimen rather than the control specimen.

These facts described above may indicate that there is the functional pre-cancerous stage of gastric secretion, on which further investigation will be made.

Conclusion

The differences of high-molecular substances of gastric juice between the healthy acidic controls and the cancerous or the anacidic specimens in chronic pathological conditions were studied as to their Pfw-activities, electrophoretical patterns and chemical compositions.

Pre-cancerous changes in the constitutions of gastric juice were suggested from the observation that the anacidic specimen in chronic pathological condition had a tendency to resemble, in the nature, the cancerous specimen rather than the healthy control specimen.

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要　旨

第1報　胃液高分子組織の Polarograph 的，沪紙電気泳動的，並びに化学的研究

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細川　忍，森本義雄，吉川春夫
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有酸対照胃液・histamine 抵抗性無酸胃液および胃癌胃液について，その polarograph 的(P)，電気泳動的，並びに diphenylamine (DPA)・tyrosine (Tyr)・hexosamine (Hxm)・hexuron 酸 (Hxu)・nitrogen (N) 等の化学的測定値の比較検討を行った。

P 的には胃癌胃液は他のものに比し波高の変動が最も著しく，また insulin 静注後 20 分目に波高の低下を来し，これは診断上有用である。

化学的測定の含量はすべてが胃癌，histamine 無酸，有酸対照例の順に高い。これらの化学的側定値の中，DPA と Hxm, Tyr と N がそれぞれ相関する。DPA-Hxm と Tyr-N 系とは相互に相関はない。したがって胃癌胃液で増量する高分子組成は DPA-Hxm 系と Tyr-N 系の二種に分つことが出来る。

Glass and Boyd 法に従って胃液を分画すると，有酸胃液では泳動的に易動度の最も速い mucoprotein 分画に P 活性が高く，一方胃癌胃液では易動度の遅い mucoproteose 分画に P 活性は移行し同時に高い DPA, Hxm 含量を認める。histamine 無酸胃液では P 活性度の移行，泳動像上の態度，並びに化学測定値の上で，すべてが有酸対照と胃癌胃液との中間的態度を示す。

その詳細については後報したい。

（文部省科学研究費による）

[GANN, 49, 261~270; December, 1958]

PART II. ELECTROPHORETICAL STUDIES ON FRACTIONATED GASTRIC JUICE —PERTAINING TO HISTAMINE REFRACTORY ANACIDIC SPECIMEN—

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In the previous paper[1], electrophoretical and chemical analyses in several gastric disorders with special reference to polarographical characteristics were made on native gastric juices and on their fractionated specimens, using Glass and Boyd's method[2].

In the present paper, further electrophoretical studies were made on the histamine refractory anacidic specimen with several chronic gastric disorders, fractionated by Glass and Boyd's method, and compared against that of healthy control and gastric carcinoma.

MATERIALS

Histamine refractory anacidic samples (using 1.0 mg histamine subcut. inj.) were selected from chronic gastritis and chronic peptic ulcer.

Aspiration of the above gastric juice was made at intervals of 20 minutes for 1 hour, after intravenous injection of 10 units of regular insulin as described in the previous paper[1]).

The pH of each of the aspirated gastric juice samples was above 7,0. The above gastric juice samples were pooled and designated as histamine refractory anacidics.

Gastric juice in gastric carcinoma and that of healthy acidic control were pooled in the same way.

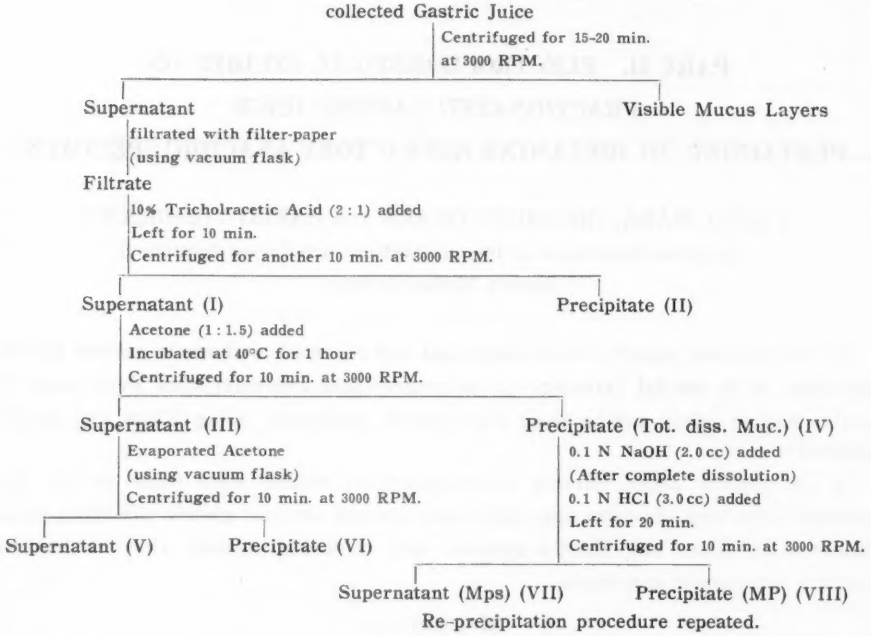
METHODS

The pooled gastric juices were subsequently filtrated to remove contaminants. The filtrates thus obtained were used in the following fractionation.

Fractionation of gastric juice: Fractionation was conducted according to Glass and Boyd's method[2]. In addition, acetone-soluble fractions were subjected to analysis as shown in Tab. 1.

Each of the fractionated specimens, introduced into a cellophane sack, was dialysed and condensed simultaneously on Carbowax 1500 at 0°-5°C. The protein

Table 1. Method for fractionation of gastric juice



concentration was brought to 3 per cent by means of biuret colorimetry. Paper electrophoresis was carried out with these specimens.

Paper electrophoresis: Paper electrophoresis was carried out in both veronal buffer (pH 8.6 μ=0.06, 10 V/cm) and in acetate buffer (pH 4.5 μ=0.08, 10 V/cm) for 4 hours.

Staining: Stains by amidoblack 10 B[3] and periodic acid Schiff (PAS)[4] method were made in the same electrophoretic paper.

Results

A. Electrophoretic patterns in native gastric juice

Electrophoretic patterns of 3 groups (histamine refractory anacidics, carcinomatous and healthy acidic controls) are shown in Fig. 1.

The patterns at pH 8.6, in all 3 groups, consisted of 5 main peaks, which were named B_1 to B_5 peak by the present authors, counting from the fastest peak towards the original point. And further 2 or 3 small peaks were usually noticed at B_2 and B_3 peak.

B_1 and B_4 peak are markedly pronounced, while a small formation in midportion, i. e., B_2 and B_3 was observed in healthy control group.

On the other hand, diminution of B_1 peak and large peaks coinciding with B_2

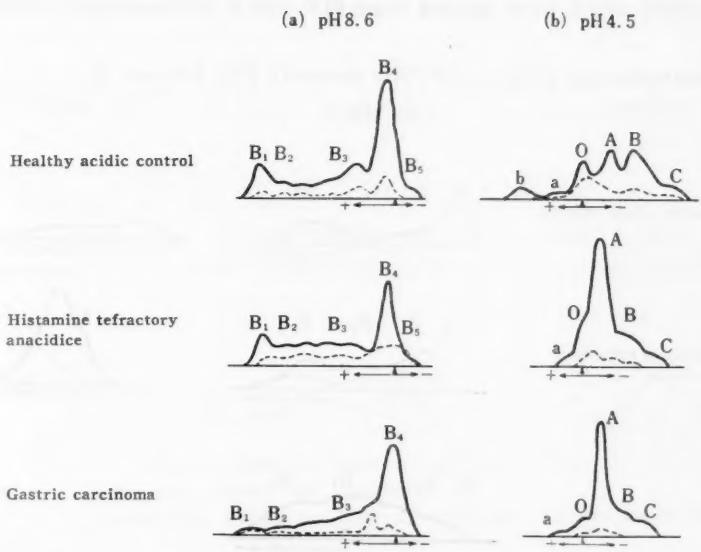


Fig. 1. Paper electrophoretic patterns of native gastric juice (FI).
Solid line: stained with amidoblack 10 B.
Dotted line: stained with periodic acid schiff stain (PAS).

and B_3 peak were observed in carcinomatous group.

In the electrophoretic pattern of histamine refractory anacidic group, an increase in B_2 and B_3 peak, and an increase in B_1 peak were observed simultaneously.

PAS-stained patterns of these groups are shown in Fig. 1 a) and b) appear as a dotted line.

In the electrophoretic pattern at pH 4.5 of healthy control (Fig. 1 b), 2 peaks a and b at anode, 3 peaks A, B and C at kathode were noticed on each side of the original point (O peak). On the other hand, b peak is seen in neither the electrophoretic pattern of cancerous specimen nor in the histamine refractory specimen at pH 4.5.

In the PAS-stained pattern, although intensive stain was seen in O peak and adjacent portions, the difference between each specimen was not marked.

B. Electrophoretic patterns in fractionated gastric juice

Each fractionated specimen was named, as shown in Tab. 1, FI is native gastric juice, FII is a residue of the former, and FIII was impossible to be studied electrophoretically due to the fact that the fraction is inevitably fractionated into FV and VI.

Electrophoretical analysis was, therefore, carried out in the following fractions, FIV containing total dissolved mucin as designated by Glass-Boyd[5], FVII (Ms)

and VIII (MP) which were isolated from FIV, and 2 acetone-soluble fractions FV and VI.

The electrophoretic pattern of FIV is shown in Fig. 2-a and -b.

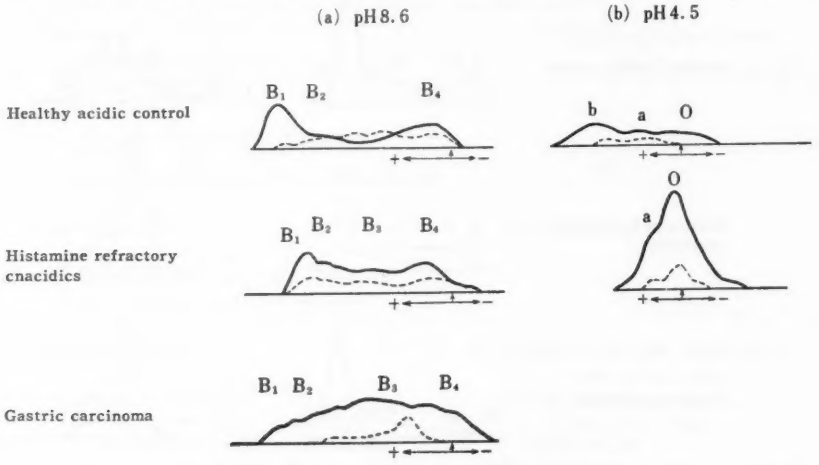


Fig. 2. Paper electrophoretic patterns of FIV.
Solid line: stained with amidoblack 10 B.
Dotted line: stained with periodic schiff stain (PAS).

FIV of control group consisted of B_1 and B_4 peak at pH 8.6 electrophoretically, while this fraction consisted of 3 peaks, O-, a- and b- peak at pH 4.5.

Contrary to the control, ill-defined peaks running from B_1 to B_4 at pH 8.6 and 2 peaks of a and O at pH 4.5 were seen in the electrophoretic pattern of histamine refractory specimen. In the carcinomatous specimen, a single and a large peak spreading from B_1 to B_4 with respect to its mobility was observed.

FVII of control group consisted of a single B_4 peak at pH 8.6 and a low O peak at pH 4.5 as is shown in Fig. 3. In the histamine refractory anacidic group, as well as in carcinomatous group, 2 peaks of B_3 and B_4 were noticed at pH 8.6 and an O peak was seen at pH 4.5. These peaks are higher than the corresponding peaks of control group.

In FVIII (MP) of control group, a single large B_1 peak (pH 8.6) and well-defined peaks of b-, a-, and O-peak (pH 4.5) were observed electrophoretically. (Fig. 4)

On the other hand, in the histamine refractory anacidic group, B_1 and B_2 (pH 8.6), and a and O (pH 4.5) were noted. Hardly any peaks were formed in the carcinomatous group at pH 8.6, and a small O peak was seen at pH 4.5.

The electrophoretic pattern shown in FV of all groups are high, as is seen in Fig. 5, and are similar to that of native gastric juice. All peaks with the exception of B_1 (pH 8.6) and of b and a peak (pH 4.5) were observed in this fraction of

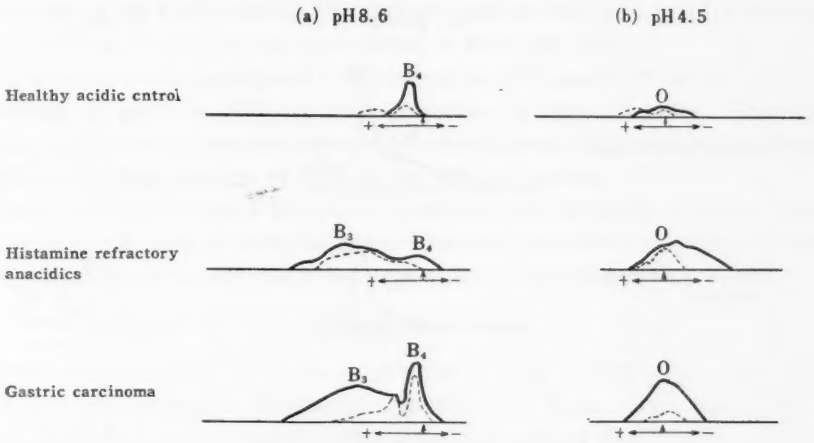


Fig. 3. Paper electrophoretic patterns of FVII.
Solid line: stained with amidoblack 10 B.
Dotted line: stained with periodic schiff stain (PAS).

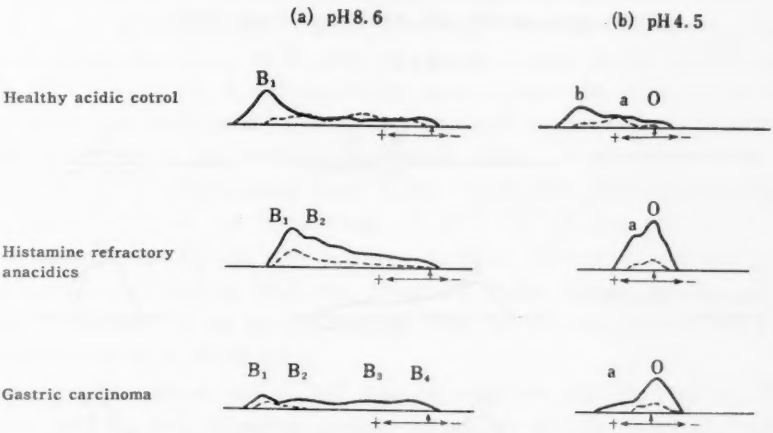


Fig. 4. Paper electrophoretic patterns of FVIII.
Solid line: stained with amidoblack 10 B.
Dotted line: stained with periodic schiff stain (PAS).

control group. FV of the histamine refractory anacidic group consisted of B_3 and B_4 at pH 8.6, and consisted of A, B and C at pH 4.5. This fraction of carcinomatous group is electrophoretically similar to the histamine refractory group. Therefore, it is suggested that a great part of the components with the exception of B_1 (pH 8.6) or a and b (pH 4.5) is transferred into this fraction.

In the electrophoretic pattern of FVI (Fig. 6), B_2 peak is mainly found at pH

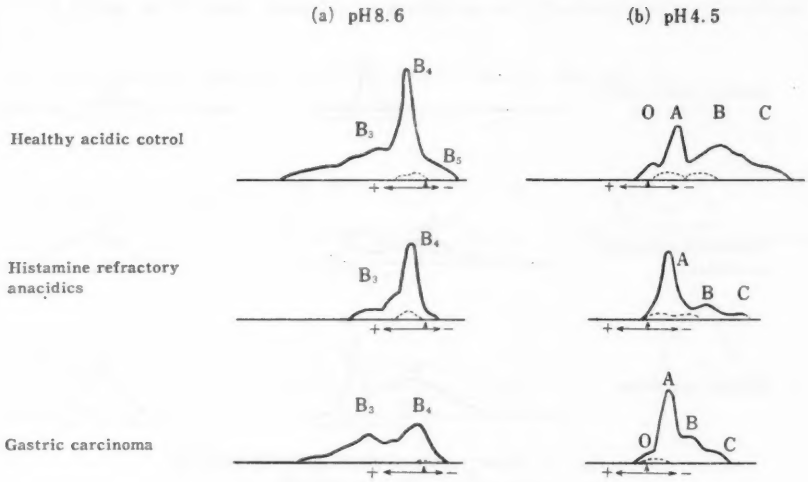


Fig. 5. Paper electrophoretic patterns of FV.
Solid line: stained with amidoblack 10 B.
Dotted line: stained with periodic schiff stain (PAS).

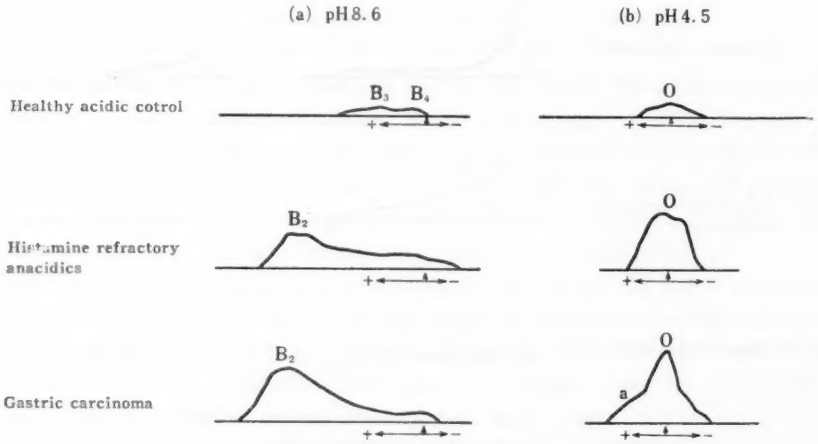


Fig. 6. Paper electrophoretic patterns of FVI.
Solid line: stained with amidoblack 10 B.
Dotted line: stained with periodic schiff stain (PAS).

8.6 in both histamine refractory and cancerous specimen, and an O peak is seen in histamine refractory anacidics, while O and a-peak is found in cancerous specimen at pH 4.5. In control specimen, no detectable peak is formed at pH 8.6 or at pH 4.5 in contrast to the findings of the above mentioned two groups.

In regards to the PAS-staining, PAS-stained positive substance, in all 3 groups, was not seen in FV and VI, but was noticed in FVII and VIII.

In comparison to the findings of PAS-staining in FVII and VIII to each group, the density of stain in FVII is more intenstive in both histamine refractory anacidics and cancerous specimen than in the control group. PAS-staining increases in intensity with the increase of FVII in the said two groups.

In FVIII of PAS-staining, PAS-positive substance was detectable in the portion coinciding with B_1 peak in both histamine refractory anacidics and carcinomatous group, whereas no detectable stain was seen at B_1 of control group.

Discussion

Various investingations were made relating to the electrophoretic study of gastric juice. Grossberg[6], Henning[7),8)], Mack[9)], and Pugh, Glass and Wolf[10)] reported that the human or canine gastric juice consisted of 4 or 5 peaks electrophoretically. The same results have been obtained in the present paper.

Comparing the electrophoretic patern of healthy control gastric juice with that of pathological cases, a diminution of B_1 peak and increase of B_2 and B_3 peak was observed in carcinomatous gastric juice, whereas is histamine refractory anacidic gastric juice no diminution of B_1 peak was noted in spite of the increase of B_2 and B_3 as was observed in carcinomatous gastric juice. In other words, it is interesting to note that histamine refractory anacidics is placed electrophoretically between acidic control and carcinomatous gastric juice.

Concerning the electrophoretical study on the fractionated gastric juice according to the method of Glass and Boyd, Pugh, et al.[10)] reported that MP showed fast negative mobility and Ms has slow negative mobility. The result obtained by the present authors, coinciding with the report by Pugh, shows that MP (FVIII) migrates to B_1 peak at pH 8.6 and to a- b- peak at pH 4.5, whereas Ms (FVII) forms a single B_4 peak at pH 8.6.

Summarizing the above results, MP fraction coincides with B_1 peak at pH 8.6 and simultaneously with a- and b- peak at pH 4.5, on the other hand Ms fraction migrates to B_4 peak at pH 8.6 and simultaneously to O peak at pH 4.5.

On the electrophoretic pattern of FV and VI that hitherto has gone unnoticed, it was clear that FV is not electrophoretically homogenous, because this fraction has all peaks with the exception of B_1 (pH 8.6) or a- and b- peak (pH 4.5). It is therefore postulated that all constituents of gastric juice with the exception of MP are contained in this fraction.

Comparing FV of control group with that of the other 2 groups, electrophoretically higher density is observed in both histamine refractory anacidics and cancerous specimen than in the control specimen, which coincided with the findings

of chemical contents reported in the previous paper[1].

On the other hand, a small B_2 and B_3 (pH 8.6) and also a small peak at the original point (pH 4.5) was observed in FVI of control. The highest peak of the 3 groups is seen, in cancerous specimen, also in this fraction as well as in FVII, which is seconded by histamine refractory anacidic specimen, with control as the lowest.

In a review of the above results, particularly in histamine refractory anacidic specimen, B_2 peak besides B_1 was observed in the electrophoretic pattern of FVIII isolated as MP, and O and a-peak was observed at pH 4.5 in addition to the finding of control specimen. In FVII fractionated as Ms, B_3 peak is accompanied by B_4 (A, B and C peak at pH 4.5) were high, and a single B_2 peak (O and a-peak at pH 4.5) was observed in FVI.

When the above findings of anacidic specimen are compared with the control pattern, it seems likely that the increase of FV, VI and VII has resulted from an increase in B_2 and B_3 peak. This finding ressembles that of carcinomatous pattern.

It is also possible to say that the electrophoretic heterogenity of fractionated specimens, not to speak of B_2 peak moving partially into FVIII, is due to the fact that the fractionation can not be made as was suspected in the control specimen, probably because of the complexity of its constituents.

Moreover, in the observation of PAS-staining, it may be also said that histamine refractory anacidic specimen is similar to cancerous specimen, from the fact that PAS chromogen is found to exist at the corresponding portion with B_1 peak contrary to the control pattern.

Two acetone-soluble fractions, i. e., FV and VI, should not be disregarded at least in pathological gastric juice, since the relative increase and the electrophoretic heterogenity of these fractions were observed in both histamine refractory anacidic specimen and carcinomatous specimen, which coincides with the results as reported in the previous paper[1], which the increase of several chemical contents including DPA chromogen was seen in these fractions of pathological gastric juice.

The present results show that MP is distinctly fractionated in the control specimen, while on the other hand, Ms increases heterogenously in pathological specimen. This may support Glass and Boyd's view[5),11),12] that the former is a physiologic constituent and the latter is a destructive product of gastric juice.

It is likely, furthermore, that B_1 peak (at pH 8.6) coincides with a- and b- peak (pH 4.5), B_2 peak with O peak, and B_3-, B_4-, B_5- peak with A-, B-, C- peak repectively, in comparison to the electrophoretic pattern at pH 8.6 with pH 4.5.

Summary

In comparison to the histamine refractory anacidic specimen of several gastric disorders with acidic control and with carcinomatous specimen, the following conclusions were obtained by electrophoretical investigation in both native gastric juice and in fractionated specimens according to the method as reported by Glass and Boyd.

1. The electrophoretic pattern in native gastric juice of histamine refractory anacidics was placed between that of healthy control and carcinomatous gastric juice. That is to say, the electrophoretic pattern of healthy acidic control shows B_1 and B_4 peak clearly, as compared with B_2 or B_3 peak. The pattern of carcinomatous gastric juice differs from the control pattern in two points, showing that an increase of B_2 and B_3 peak and diminution of B_1 peak were present.

On the other hand, in the histamine refractory anacidics, increase of B_2 and B_3 peak is seen as in the case of carcinomatous specimen, whereas B_1 peak is clearly observed as in the case of the control.

2. In the fractionated specimen, FVIII (MP) is closely connected with B_1 peak (at pH 8.6) or a- and b- peak (at pH 4.5) electrophoretically. FVII (Ms) is connected with B_4 peak (at pH 8.6).

B_2 and B_3 peak, which increased in both histamine refractory anacidics and cancerous specimen, seemed to be connected with FVI and FVII. It is likely that the increase in FV, VI and VII corresponds with the above pathological patterns.

Moreover, it was ascertained that the both fractions of MP (FVIII) and Ms (FVII) in pathological gastric juice consisted of heterogenous components electrophoretically.

3. A considerable part of the constituents in gastric juice with the exception of MP is contained in FV, which is a supernatant of acetone-soluble fraction. And these constituents are transferred largely into FV in the case of pathological gastric juices, i. e., histamine refractory anacidics and carcinomatous gastric juices. Accordingly, it is postulated that acetone-soluble fraction can not be neglected in the pathological gastric juices.

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要　旨

第2報　胃液の沪紙電気泳動的研究
——特に histamine 抵抗性無酸胃液について

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前報に引続き特に histamine 抵抗性無酸胃液について，さらに詳細にその全胃液並びに Glass-Boyd 法による各分画の電気泳動的研究を行った。

全胃液の電気泳動像では，histamine 無酸群の態度は有酸対照および胃癌胃液の中間的態度を示す。すなわち有酸対照泳動像は易動度の速い峯 (B_1) と遅い峯 (B_4) が著明で中間易動部 (B_2, B_3) の形成は少く，胃癌胃液では B_1 が著減し B_2, B_3 が増量する。これに対し histamine 無酸群では癌同様 B_2～B_3 の増量をみるが，一方対照同様 B_1 は減少しない。

分画試料については，FVIII (MP) は B_1 (pH 8.6) 又は a, b-峯 (pH 4.5) と密接に関連し，FVII (Ms) は B_4 (pH 8.6) と関連する。中間易動部 (B_2～B_3) は主として FVI および VII と関連する。histamine 無酸および癌胃液の MP (FVIII), Ms (FVII) 分画は泳動的に均一でない。

Acetone 可溶部である FV は MP を除くすべての胃液組成を含み均一でない。また histamine 無酸および胃癌胃液では FVI に移行する部分も多い。したがって病的胃液においては acetone 可溶分画は無視出来ぬ部分と思われる。　（文部省科学研究費による）

[GANN, 49, 271~280; December, 1958]

PART III. POLAROGRAPHICAL, ELECTROPHORETICAL AND BIOCHEMICAL STUDIES ON FRACTIONATED GASTRIC JUICE BY CONTINUOUS PAPER ELECTROPHORESIS, WITH SPECIAL REFERENCE TO THE FUNCTIONAL PRECANCEROUS STAGE OF GASTRIC JUICE

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A characteristic change in high-molecular constituents of gastric juice with gastric carcinoma, using polarographical, electrophoretical and biochemical analytical methods, was noted in our previous report[1].

Since the ultimate aim of our studies is in the early detection of gastric carcinoma, it is our hope that a definite and pathological stage resembling carcinomatous gastric juice, in other words, a precancerous stage in tne secretory function of the stomach will be discovered by intensive clinical investigations.

As it was impossible to isolate an electrophoretically homogenous specimen by the previous method[2], it was also impossible to investigate the characteristics of pathologically increased constituents thoroughly. In the present study, biochemical analysis of electrophoretically homogenous fractions isolated by means of continuous electrophoresis were conducted.

Materials

Histamine refractory anacidic samples were selected from several patients with chronic gastritis or chronic peptic ulcer, and were compared with healthy acidic control and with gastric carcinoma samples.

Fasting gastric juice was removed through a small tube prior to histamine stimulation, in order to avoid any influence by saliva contamination. Gastric juice was aspirated at 20 minutes intervals for 1 hour following a histamine injection of 1 mg, and the acidity of each gastric juice was measured by usual clinical method and by means of glass-electrode ionimater.

Gastric samples showing more than pH 5.0 in each specimen were pooled and designated as histamine refractory anacidic sample. Samples contaminated with bile and blood were omitted. Healthy acidic sample and carcinomatous gastric juice were simultaneously treated in the same manner.

Each gastric sample thus pooled was condensed and lyophilized after dialysis

against distilled water for 24 hours at 5°C.

Approximately 300 mg of the powder thus obtained was dissolved in 6 ml with the same veronal buffer solution (pH 8.6, μ=0.03) as used in electrophoresis. The protein concentration was brought to 3 per cent by means of biuret colorimetry.

Finally, the sample thus obtained was fractionated by means of continuous electrophoresis.

Methods

Continuous paper electrophoresis procedure:

An apparatus modified by the authors was used in this procedure.

The paper used in electrophoresis was Tōyō no. 52 (26×26 cm).

Veronal buffer solution (pH 8.6, μ=0.03) was used in the present study.

Continuous electrophoresis was performed under the following conditions; Constant voltage 225 V, 7.5–8.25 mA, 24 hours duration. In order to lead the sample to the paper, a wick of 1.5 mm diameter was used.

Strip paper electrophoresis:

An aliquot part of sample was simultaneously studied by means of paper electrophoresis (pH 8.6, μ=0.06), and compared against the continuous electrophoretic pattern.

Physico-chemical analyses:

Each of fractionated specimens obtained by continuous electrophoresis was brought to an equal volume of 10 ml with veronal buffer mentioned above and was analysed in the following manner.

1) Polarographic method: Direct[3] and filtrate test[4] (20% sulfosalicylic acid) were made.

2) Orcinol reaction: Using Bial's orcinol reagent, optical density was measured at both 575 mμ and 680 mμ according to Svennerholm's method[5]. The content of Orcinol chromogen was expressed in values representing the difference between the above 2 densities.

3) Diphenylamine (DPA) reaction: The content of diphenylamine chromogen was measured by Niazi and State's method[6].

4) Tyrosine (Tyr) content: Using phenol reagent, tyrosine content was determined according to the method of Glass and Boyd[7].

5) Hexosamine (Hxm) content: The content was determined as Glucosamine by Shida's modification[8] of Elson-Morgan's method[9].

6) Hexuronic acid (Hxu) content: Using α-naphthoresorcinol picrate, the content was determined as glucuronic acid according to the method of Ishidate and Nanbara[10].

7) Nitrogen (N) content: The content was measured by Microkjeldahlometry.

Results

Healthy acidic control: Fig. 1 shows one instance of continuous electrophoretic and strip electrophoretic patterns.

Continuous electrophoretic pattern and chemical contents of fractionated specimens in healthy acidic control are shown in Tab. 1 and Fig. 2.

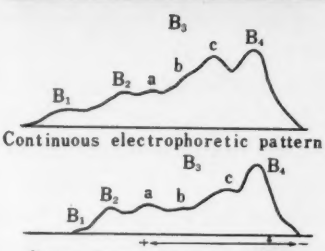

Fig. 1.

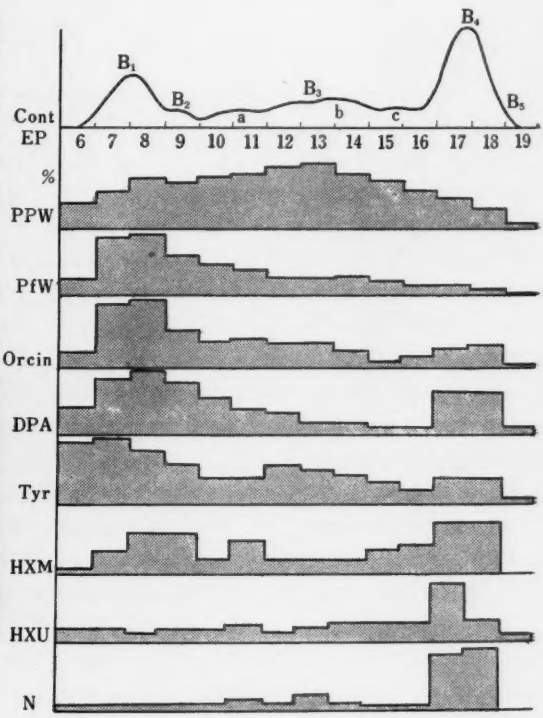

Fig. 2. Continuous electrophoretic pattern and several physico-chemical values of fractionated gastric juices with healthy acidic control.

Numbers under the electrophoretic pattern represent the respective number of collecting-tubes containing fractionated specimens. Polarographical and chemical values of each fractionated specimen are indicated in percentage.

Table 1. Physico-chemical assays in fractionated specimens of healthy acidic control

	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Polarographic protein waves	13	28	50	43	50	52	58	61	50	45	38	31	12	6
Polarographic filtrate waves	12	50	52	45	35	21	18	18	20	18	13	12	10	4
Orcinol reactive substance 575 mμ	0.047	0.100	0.150	0.115	0.095	0.100	0.085	0.065	0.055	0.045	0.035	0.065	0.065	0.005
(Extinction) 680 mμ	0.032	0.040	0.080	0.070	0.065	0.050	0.055	0.035	0.035	0.035	0.020	0.040	0.038	0
values	0.015	0.060	0.070	0.045	0.030	0.050	0.030	0.030	0.020	0.010	0.015	0.025	0.027	0.005
Diphenylamine reactive substance (Extinc.)	0.040	0.077	0.098	0.089	0.065	0.059	0.060	0.033	0.033	0.030	0.030	0.064	0.064	0.028
Tyrosine content (mg%)	0.097	0.106	0.080	0.059	0.052	0.052	0.058	0.056	0.052	0.043	0.034	0.054	0.054	0.020
Hexosamine (mg%)	0	3.5	7.5	7.5	3.5	5.5	3.5	3.5	3.5	7.5	7.5	7.5	7.5	0
Hexuronic acid (mg%)	0.88	0.74	0.50	0.76	0.76	0.88	0.44	0.76	0.96	1.00	1.00	2.40	1.00	0.40
Nitrogen (g/dl)	0.022	0.022	0.022	0.022	0	0.033	0	0.044	0.011	0.011	0.011	0.088	0.099	0

Nomenculature of the peaks in the electrophoretic pattern was designated as was reported in the previous paper[2].

Though polarographic protein wave (PPW) by means of direct test is high in the portion with moderate mobility, i. e., B_2 and B_3 peak, the highest activity of polarographic filtrate wave (Pfw) was noticed in the B_1-corresponding specimen, as is shown in Fig. 2. Orcinol reactive substance is highly contained in B_1 peak.

The content of diphenylamine (DPA) reactive substance is similar to the former, but it differs from the fomer in that it shows a relatively high content in the B_4-corresponding specimen.

The highest tyrosine (Tyr) content was observed in B_1-corresponding specimen.

Hexosamin (Hxm) content was found to be high in the portion between B_1 and B_2 and the highest in B_4.

Hexuronic acid (Hxu) content was high in the B_4-corresponding specimen.

Nitrogen (N) content was found to be high in both B_3 and B_4.

In summary, the B_1-corresponding specimen showed high PfW, high contents of orcinol reactive and DPA reactive substance, and of Tyr. The specimen coinciding with the portion between B_1 and B_2 showed relatively high Hxm content, B_3 contains Tyr and N, and the B_4-corresponding specimen showed the highest contents of Hxm, N, Hxu, and a small amount of Tyr.

Histamine refractory anacidic sample: The increase of both B_2 and B_3 peak is noticed when compared with the control. As were seen in Tab. 2 and Fig. 3, PfW and Tyr content were observed in the B_1-corresponding specimen similar to the control, however, it should be noted that high contents of both orcinol reactive and DPA reactive substance migrate into the area between B_1 and B_2 instead of B_1 observed as in the case of the control. In addition to the above, the contents of Hxm, Hxu and of Tyr are found to exist in both the B_2- and the B_3-corresponding specimen in parallel with the increase of both B_2 and B_3. The highest contents of

Table 2. Physico-chemical assays in fractionated specimens of histamine refractory anacidics

	7	8	9	10	11	12	13	14	15	16	17	18	19
Polarographic protein wave (mm)	7	12	37	37	47	50	57	35	45	33	36	13	0
Polarographic filtrate wave (mm)	4	10	47	43	48	17	26	14	14	9	6	2	0
Oricinol reactive substance 575 mμ	0.023	0.020	0.060	0.075	0.110	0.070	0.085	0.045	0.060	0.060	0.080	0.020	0.065
(Extinction) 680 mμ	0.012	0.010	0.040	0.040	0.060	0.030	0.030	0.020	0.040	0.035	0.055	0.010	0.045
values	0.011	0.010	0.020	0.035	0.050	0.040	0.055	0.025	0.020	0.025	0.025	0.010	0.020
Diphenylamine reactive substance (Extinc.)	0.018	0.034	0.046	0.064	0.080	0.054	0.056	0.045	0.035	0.033	0.066	0.052	0.020
Tyrosine content (mg %)	0.037	0.034	0.069	0.058	0.056	0.041	0.056	0.040	0.063	0.058	0.083	0.043	0.017
Hexosamine (mg %)	0	0	3.0	3.0	3.5	2.0	5.5	3.5	3.5	3.5	7.5	7.5	0
Hexuronic acid (mg %)	0	0	0.68	0.76	0.88	0.84	1.60	0.84	0.80	1.04	1.30	5.00	2.50
Nitrogen (g/dl)	0	0	0	0	0.056	0.056	0.056	0	0.084	0.056	0.168	0.084	0.056

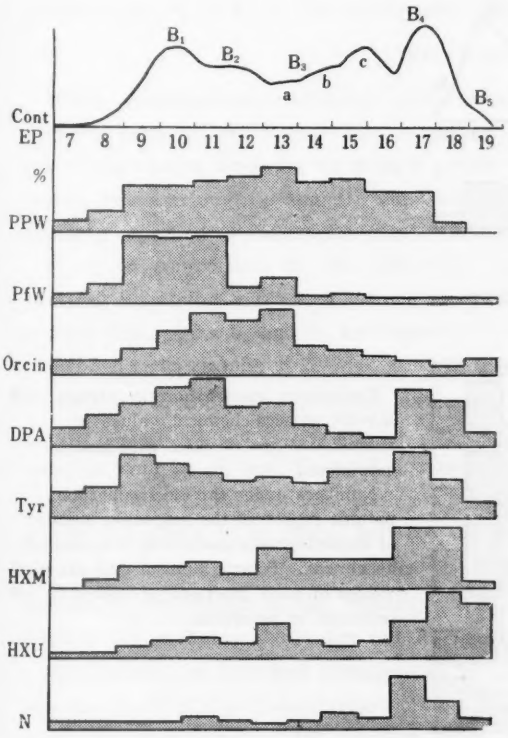

Fig. 3.
Continuous electrophoretic pattern and several physico-chemical values of fractionated gastric juice with histamine refractory anacidics.
Numbers under the electrophoretic pattern represent the respective number of collectingtubes containing fractionated specimens. Polarographical and chemical values of each fractionated specimen are indicated in percentage.

N, Hxm and of Hxu are observed in B_4 similar to the control.

Carcinomatous gastric juice (Tab. 3 and Fig. 4): Diminution of B_1 and a remarkable increase of both B_2 and B_3 were noticed coinciding with the finding of

Table 3. Physico-chemical assays in fractionated specimens of Gastric carcinoma

	7	8	9	10	11	12	13	14	15	16	17
Polarographic protein waves (mm)	19	25	49	53	59	65	70	54	49	57	21
Polarographic filtrate waves (mm)	12	19	39	35	31	28	23	14	9	11	4
Orcinol reactive substance 575 mμ	0.060	0.050	0.180	0.132	0.120	0.092	0.054	0.040	0.052	0.086	0.055
(Extinction) 680 mμ	0.032	0.021	0.078	0.072	0.071	0.050	0.039	0.031	0.040	0.060	0.035
values	0.028	0.029	0.102	0.060	0.049	0.042	0.015	0.009	0.012	0.026	0.020
Diphenylamine reactive substance (Extinc.)	0.060	0.072	0.149	0.159	0.118	0.098	0.061	0.052	0.072	0.183	0.115
Tyrosine content (mg %)	0.038	0.034	0.053	0.057	0.051	0.049	0.057	0.062	0.130	0.177	0.086
Hexosamine (mg %)	5.0	5.0	7.5	8.0	11.5	8.0	8.0	11.5	18.5	27.5	13.0
Hexuronic acid (mg %)	0.8	0.4	1.5	1.1	0.9	0	0	1.1	2.5	9.7	3.5
Nitrogen (g/dl)	0	0.146	0.146	0.146	0.146	0.146	0.146	0.146	0.366	0.585	0.731

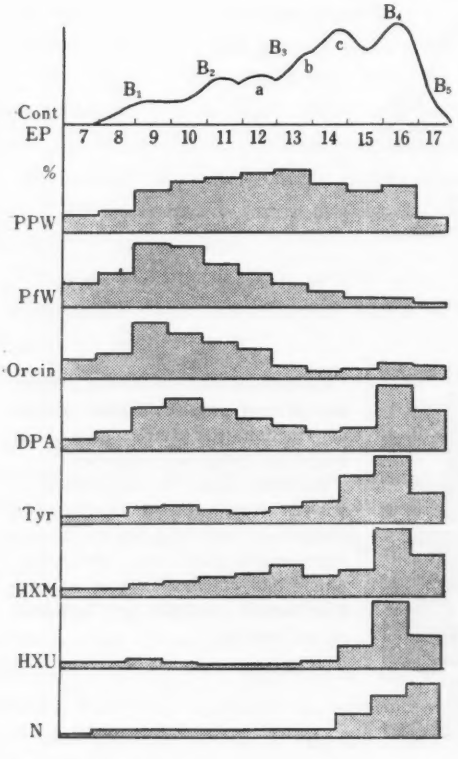

Fig. 4.
Continuous electrophoretic pattern and several physico-chemical values of fractionated gastric juice with gastric carcinoma.
Numbers under the electrophoretic pattern represent the respective number of collectingtubes containing fractionated specimens. Polarographical and chemical values of each fractionated specimen are indicated in percentage.

previous paper[2].

It is of interest to note that high PfW and high contents of orcinol reactive and DPA reactive substance, which were observed in B_1 of the control, widely

migrated into the area between B_1 and B_2 or into B_3, also the highest Tyr content was observed in B_4 instead of B_1. The contents of N, Hxm and of Hxu were noticed in B_4 similar to the control.

It is noteworthy, from the above finding, that the relatively increased fraction in pathological gastric juice is both B_2 and B_3, and that the increment of the said peaks is the highest in gastric carcinoma, which is followed by histamine refractory anacidic sample with the lowest in the healthy control, in other words, the histamine refractory anacidic sample is placed between cancer and control.

As regards the biochemical characteristics of pathologically increased specimens, both contents of orcinol and DPA chromogen are increased in these specimens.

That is to say, the above 2 chemical contents are observed in B_1 in the control, whereas they have a tendency to migrate into the portion with slower mobility in the histamine refractory anacidics, and come to be noticed in widely spread portion from B_2 to B_3, in the carcinomatous specimen.

Discussion

When continous electrophoretic patterns are compared with the strip electrophoretic patterns, the former was in general analogous with the latter, although the former has a tendency to form a gently sloping B_1 peak because of its developing into a triangular shape. Thus the fractionated specimens obtained may be possibly regarded as electrophoretically homogenous.

First, in comparison to electrophoretic patterns of three groups, i. e., acidic control, histamine refractory anacidics and carcinomatous gastric sample, well defined high peaks B_1 and B_4 are observed in contrast with low B_2 and B_3 in the control gastric sample.

Contrary to the above findings, extremely low B_1 and increase of both B_2 and B_3 are noticed in the carcinomatous gastric sample. In the histamine refractory anacidic sample, B_2 and B_3 peak increase as in the case of the carcinomatous sample, while high B_1 and B_4 peak are noted as in the case of control. In other words, it appears to be placed between the above two samples.

From the above findings, it is clear that the increase of both B_2 and B_3 is not caused by the change of the mobility of B_1 peak accompanied with the lack of acidity, but probably shows the relation with their pathological conditions.

In a survey of chemical constituents in each fractionated specimen, at first, B_1 is a low in N and polysaccharide-rich component, which most certainly coincides with the mucoprotein (MP) fraction from the fact that it shows a high PfW activity.

The fact agrees with the previous findings[2] obtained by the method of Glass and Boyd's fractionation. A diminution of B_1 in the carcinomatous sample is due

to the decrease of MP content; which decrease was reported by many investigators[11),12),13)].

In the histamine refractory anacidic sample, B_1 peak is found to exist in spite of the lack of HCl secretion.

This fact indicates that both MP and HCl are not always secreted together in gastric juice, in parallel.

Secondly, both B_2 and B_3 peak are low in the control, but are increased in pathological samples electrophoretically. These peaks appear to contain a PPW active substance. As regards the characteristics of pathologically increased B_2 and B_3 peak, it should be noted that orcinol and DPA reaction are exhibited highly with these peaks instead of B_1 peak as in the case of the control. The increase in the contents of Hxm, Hxu and of Tyr is also observed in these peaks of the pathological samples.

It might be assumed that the denaturated substances come to be detected in these peaks with moderate mobility, probably owing to the fact that the protein moiety of pathological samples are easily decomposed. This speculation coincides with the findings of the previous paper[2)] in which was reported that FV and VI other than mucoproteose (Ms) fraction (FVII), in the experiment with the Glass and Boyd fractionation, would be responsible for the increase in B_2 and in B_3 of the pathological gastric juices.

Lastly, the B_4-corresponding specimen contains a relatively constant chemical constituents as well as B_1, namely, the said specimen contains high N, Hxm, Hxu and Tyr. These contents are in parallel with the increase or decrease of electrophoretic pattern. No significant difference exists among the said three groups of gastric samples.

The present result agrees with the previous findings[2)] in which it was reported that B_4 peak mainly consists of Ms fraction from the electrophoretical study on the fractionated gastric juice. The B_5-corresponding specimen seems to be not characteristic.

Summarizing the above, it can be assumed that the histamine refractory anacidic sample is placed between the acidic control and the carcinomatous gastric sample from the fact regarding the electrophoretic pattern and the transition of chemical constituents in the fractionated specimens.

The problem as to whether the increase of B_2 and of B_3 noticed in the anacidics is related to the characteristics in the secretory function which corresponds with the functional precancerous stage, or whether it is related to the indirect change of high-molecular constituents accompanied with the defect of hydrochloric acid secretion, should be pursued hereafter by extensive investigations of numerous cases based on the above views.

Thus a new method of early diagnosis of gastric carcinoma by investigating the details of increased fractions in carcinomatous specimen, may be expected regardless of whether the increased fractions happen prior to the growth of carcinoma or not.

Furthermore, histological investigations in the histamine refractory anacidic sample are in progress, in parallel with the study of relationship between pathologically increased fractions and the ability of hydrochloric acid secretion.

Summary

1. Polarographical waves and biochemical contents of orcinol reactive, diphenylamine reactive substance, glucosamine, glucuronic acid, tyrosine and of nitrogen were measured on fractionated specimens by means of continuous electrophoresis with regards to three groups of gastric juices, the healthy acidic control, the gastric carcinoma and the histamine refractory anacidic sample.

2. Electrophoretic patterns obtained by continuous electrophoresis agree with that of strip paper electrophoresis. Thus, it was ascertained that the above chemical analyses were performed on electrophoretically homogenous specimens.

3. In the acidic control, the highest polarographical filtrate wave and the highest contents of orcinol chromogen, diphenylamine chromogen, and of tyrosine were observed in B_1 peak with the fastest mobility. Hexosamine content was highest in B_4, and was also found to exist in the area between B_1 and B_2. The contents of hexuronic acid and nitrogen were noticed in B_4 with slow mobility.

4. In both histamine refractory anacidics and carcinomatous gastric juice, it was found that the above chemical contents observed in B_1 were transferred into B_2 or B_3. And it was of interest to note that the histamine refractory anacidic sample was placed between the acidic control and the carcinomatous sample with respect to their electrophoretic patterns and characteristic behaviours of chemical constituents. Thus the clarification of functional precancerous stage may be expected by investigating chemical constituents, in detail, which are related to B_2 and B_3.

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要　旨

第3報　連続沪紙泳動法による分画胃液の Polarograph, 電気泳動的，並びに生化学的研究 ——特に胃液の機能的前癌性変化について

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これまでの知見を今回は連続沪紙泳動法を用い，有酸対照例・胃癌並びに histamine 抵抗性無酸胃液を分画し，polarograph 法・orcinol 反応・diphenylamine 反応・hexosamine・hexuron 酸・tyrosine および nitrogen 含量を測定した。

連続沪紙泳動像と平面沪紙泳動像とは極めてよく一致した像を示す。したがって上述の理化学的測定値は泳動的に均一な分画についての分析値と考えられる。

有酸対照胃液では，Polarograph 波・Orcin・DPA・Tyr 含量は易動度の最も速い B_1 に多く認められ，Hxm は B_1 と B_2 の中間および B_4 に，Hxu, N は B_4 に最も多く認められた。

Histamine 無酸胃液および胃癌胃液では，上記理化学分析値の含量が次第に易動度の遅い B_2, B_3 分画内に移行し，無酸例は有酸対照と胃癌の中間的態度を示した。

したがって今後 B_2 および B_3 分画内組成を詳細に追求することにより，機能的前癌性変化の解明が期待されよう。

（文部省科学研究費による）

[GANN, 49, 281~286; December, 1958]

PAPER ELECTROPHORETIC STUDIES ON ENZYMES IN THE LIVER OF RATS FED 4-DIMETHYL-AMINOAZOBENZENE. I. HISTIDASE

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The quantitative determination of histidase activity in the liver of rats fed 4-dimethylaminoazobenzene (DAB) has histherto been carried out by a few investigators, Masayama et al., and Viollier. They found coincidentally that the activity of histidase fell gradually during the hepatocarcinogenesis and extremely diminished in hepatoma compared with that of normal rat liver.

The present paper deals with an extension of these results of previous workers from different standpoints to characterize the enzyme; therefore not merely quantitative but more qualitative than before. For this purpose the paper electrophoresis has been employed in these enzymatic studies. In attempt to detect possible changes in the distribution of the enzyme among the different protein fractions the hepatic tissue homogenate of DAB fed rats has been applied on the paper strip for electrophoresis.

Materials and Methods

Experimental animals.——DAB rats. Albino rats of mixed strain of our laboratory stock were employed for these studies. Male rats of about 100 g were raised on the diet containing 0.06 per cent of DAB for about 150 days, then on normal diet for more than 30 days. When those rats were autopsied, the livers showed various stages of pathological changes including hepatoma.

Rats fed on carcinogenic diet in their early experimental days (4 weeks) were also examined. The livers were normal in gross appearance but were conceived to be under the influence of fed dye.

Normal rats. Untreated rats of similar age which have been placed on normal diet throughout the time of experiment, were taken as control.

The animals were sacrificed by cervical fracture, and the liver was quickly excised and blotted on a filter paper. The hepatic tissue homogenate was made with distilled water, exactly three times the weight of tissue, and was used either without further treatment, or after fractionation by centrifugation at 15,000 r.p.m. for 1 hour to obtain supernatant.

Paper electrophoresis was then carried out with an apparatus of the moist-cham-

ber type with paper (Toyo Filter Paper No. 50) horizontally streched on a plastic frame. Before the sample was introduced, the apparatus has been left with the current turned on for 5 minutes for equilibration by using the buffer solution of 0.1M veronal acetate at pH 8.4, thereafter 0.01 ml of the above tissue homogenate or supernatant was introduced onto the paper per 1 cm of the initial line, under the system of 400 volts and the current of 2.5 ma.

After 15 hours of electrophoresis the sheet of paper was removed from the frame and laid on a glass plate with centimeter scales. According to the markes the paper was cut into segments, i.e., 20–24 cm strips towards the anode and 3 cm to the cathode from the application site. Each paper segment in size of 1×3 cm was then applied to Conway unit while wet, for the micro-determination of ammonia.

Into the outer compartment of the units were delivered 1 ml of the substrate solution of 0.01 M L-histidine (originally it was hydrochloride and was neutralized by NaOH prio to use) and 2 ml of 0.1 M phosphate buffer at pH 8.3. Into the solution mixture was put each paper segment (it was cut into smaller sizes at this moment) and overlayed with toluene. Two ml of 0.02 N H_2SO_4 was transferred into the central well of the units. Then the lids were vaseline-greased and placed in position. The whole series of units were placed in an incubator at 38°C for 20 hours. At the end of this time the digestion was interrupted by opening the units and 2 ml of Nessler's reagent was pipetted into the H_2SO_4 and the amount of ammonia was measured by photo-electric colorimeter by using the filter of 500mμ.

The datum of digestion experiment by using untreated paper of the same size soaked with solely buffer solution was employed as the blank determination and was subtracted from the above measurements.

The data were then represented graphically in order to discuss the enzyme activity without difficulty as follows: the cipher of cm, which showed the actual electromigration distance of the enzyme on the paper was taken in abscissa. On the ordinate the enzyme activity was represented in γ of ammonia nitrogen, which has evolved from the digestion mixture. When the points were plotted, the distribution of histidase on the paper was obtained as a pattern of activity.

The remaining strip of the paper was used for staining of the protein zones by bromophenol blue. The amount of dye was measured by photo-electric colorimeter (filter: 570 mμ), after elution of each paper segment, which was obtained by cutting the stained paper strip into segments in suitable size as in the case of determination of enzyme activity.

Results and Discussion

The activity pattern of histidase of normal rat liver (Figs. 1-3) presented one prominent horn-shaped peak accompanied by 2 or 3 indistinct minor maxima in

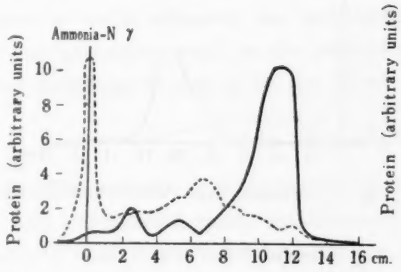

Fig. 1 Normal liver (Whole homogenate)

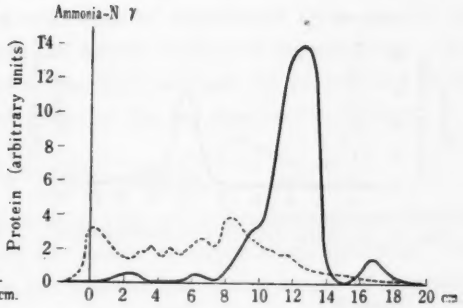

Fig. 2 Normal liver (Whole homogenate)

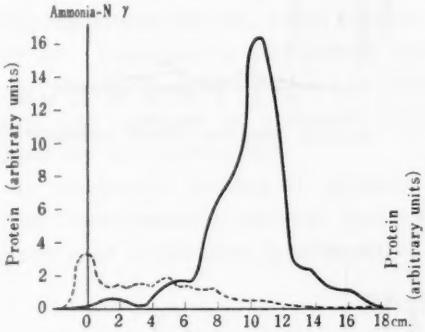

Fig. 3 Normal liver (Whole homogenate)

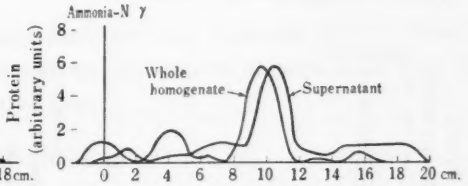

Fig. 4 Normal liver

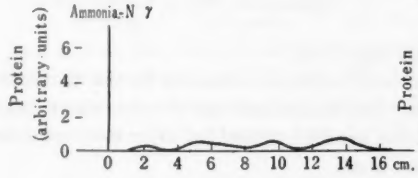

Fig. 5 Normal liver (Sediment)

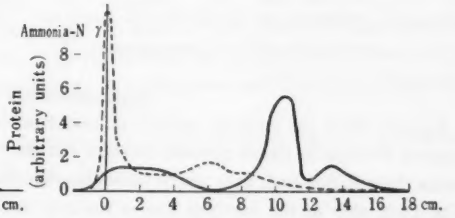

Fig. 6 Regenerating liver (Whole homogenate)

the electromigrated zone of the liver protein. An experiment was carried out to compare whole homogenate and supernatant of the same sample of normal liver and the histidase activity was measured under identical conditions. The results were represented in the similar patterns (Fig. 4). The sediment showed the trace of activity on the pattern according to the same procedure (Fig. 5).

The regenerating liver showed the pattern akin to that of the normal liver pertaining to histidase (Fig. 6). The regenerating liver was obtained from the rat,

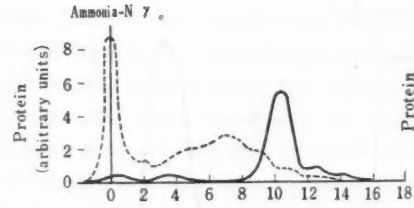

Fig. 8 Cirrhotic liver (Whole homogenate)

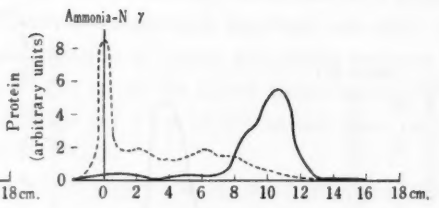

Fig. 7 Cirrhotic liver (Whole homogenate)

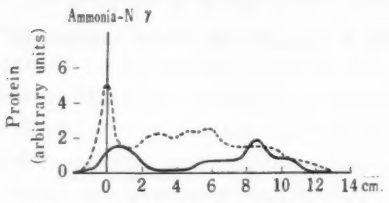

Fig. 9 Hepatoma (Whole homogenate)

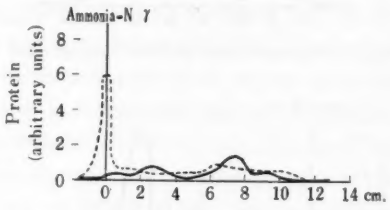

Fig. 10 Hepatoma (Whole homogenate)

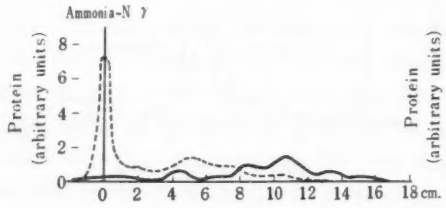

Fig. 11 DAB fed rat on its fourth week of experiment (Whole homogenate)

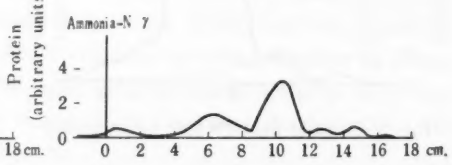

Fig. 12 DAB fed rat on its fourth week of experiment (Whole homogenate)

Explanation for Figs. 1–12

Figures show the activity pattern of histidase (solid curve) accompanied by the distribution pattern of hepatic tissue protein (broken curve). The longitudinal line of each figure represents the starting line, onto which tissue homogenate has been applied for paper electrophoresis. The right side of the starting line is towards the anode.

which had been partially hepatectomized twice with an interval of 48 hours and sacrificed at 48 hours after the second operation.

Cirrhotic liver showed the pattern having characteristic peak of somewhat lower level than that of normal liver (Figs. 7–8).

When the liver turned to cancerous, the pattern generally moved to low level, nevertheless had a convex curve at the expected site of protein distribution, where the marked peak in non-cancerous liver was found (Figs. 9–10). It might be perceived that histidase was one of the persistent enzymes, which even in cancerous

liver, still maintained its original character, although its activity was lower.

In the liver of DAB rats in their fourth week of experiment (Figs. 11–12), low patterns were obtained, but still kept the special feature of normal liver. Lack of the prominent peak on the pattern in the liver of DAB fed rat within a month was perceived to due to be the direct effect of the fed dye on the enzyme.

Summary

1) The activity of histidase in the liver of rats fed 4-dimethylaminoazobenzene (DAB) was studied rather qualitatively by means of paper electrophoresis. The activity was represented in a pattern in consideration of the length of migration of enzyme on the paper.

2) Normal rat liver showed one prominent peak on the activity pattern at the electromigrated location, which reached nearly to an end of the zone of tissue protein. Regenerating liver showed also a similar pattern to that of normal liver.

3) The liver of DAB fed rats in their early experimental days, the cirrhotic liver and hepatoma of prolonged DAB fed rats showed low activity pattern, especially low in hepatoma.

4) Qualitative changes of histidase have not been detected in the course of hepatocarcinogenesis, although the activity diminished keeping step with the progress of pathological changes of the liver.

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要　旨

バターエロー投与シロネズミの肝酵素の濾紙電気泳動的研究
第1報　ヒスチダーゼ

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従来正常組織と癌組織の酵素を比較する場合に両者の酵素活性度を単に定量的に論ずることが多かった。癌化に当って酵素がはたして本質的に母組織のその酵素と相違して現われるかどうかを沪紙電気泳動法によって追究することを企てた。

実験材料に肝癌生成物質バターエロー (DAB) を投与したシロネズミ肝を選び，ホモジエネートとし，そのままかあるいは分屑を沪紙上に電気泳動し，蛋白ゾーンの中に如何に酵素が配分されているかを観察した。

それには泳動後の沪紙を一定幅の多数片に裁断して一つ一つを酵素源とし，同数のコーンウエイ装置を準備して，基質ヒスチヂン溶液と pH 8.6 の燐酸緩衝剤の混合液から 22 時間に産生するアンモニアを定量した。この数値をグラフの縦軸にとり，横軸には切り取られた沪紙片の原点からの距離を目盛って結んで得た活性度パターンを検討した。

正常肝のヒスチダーゼ活性パターンは易泳動部に一箇の著しく突起している曲線を認めた。しかしてその位置は肝蛋白ゾーンの末端に近い部であった。正常肝のパターンと再生肝の示すものとは性格的には変りなかった。

DAB 投与4週で上記パターンの特性が殆んど失われた。これは摂取した DAB に直接影響されたものと思われる。DAB 長期投与でしかも正常食に戻してなお飼育をつづけたシロネズミの病変肝のうち，硬変肝は稍低いがパターンの特徴を示した。肝癌のパターンは最も低調であったが，痕跡ながらなお特性がうかがわれた。これらの観察からヒスチダーゼはその産生される臓器，すなわち肝の癌化に伴って漸減する活性度の量的の差は諸学者の報告と同様であったが，沪紙電気泳動法によっては性格的変化を認め得なかった。(文部省科学研究費による)

[GANN, 49, 287~294; December, 1958]

PAPER ELECTROPHORETIC STUDIES ON ENZYMES IN THE LIVER OF RATS FED 4-DIMETHYLAMINOAZOBENZENE. II. ACID AND ALKALINE PHOSPHATASE. APPENDIX: SERUM ALKALINE PHOSPHATASE OF DYE-FED RATS

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In recent years much work has been carried out by several investigators pertaining to acid and alkaline phosphatase of the rat liver during hepatocarcinogenesis, produced by 4-dimethylaminoazobenzene (DAB), the outline of which may be stated as follows: the activity of acid and alkaline phosphatase in the pathological but non-cancerous liver was similar to that of the normal rat liver. When the liver became cancerous, however, alkaline phosphatase displayed extremely high activity. DAB feeding itself appears to have no significant effect upon phosphatase activity directly.

A few enzyme was hitherto known, which displays higher activity in cancerous tissue than non-cancerous, mother tissue. Among them alkaline phosphatase was conceived as one of the noteworthy enzymes. The present author planned to investigate the phosphatase by the aid of paper electrophoresis. The idea of this experiment was similar to that described in the preceding paper about histidase.

Methods

Experimental animals.——DAB rats. The male rats weighing about 100 g of our laboratory stock were maintained on the DAB diet (0.06 per cent) for 150 days, after which the rats were removed from the DAB diet and replaced on normal diet for additional weeks. When autopsied the liver of rats showed, in gross appearance, various grades of pathological changes including hepatoma. DAB rats in their fourth week of experimental days were also employed in order to obtain the liver, macroscopically normal but disposed under the influence of fed dye directly.

Normal rats. Intact rats of similar weight which have been placed on normal diet (rice grains) throughout the time of experiment, were taken as control. Both normal and carcinogen fed rats were allowed to consume the food and water ad libitum and occasionally supplemented with dried fish and green vegetables.

Materials. Hepatic tissues.

The rats were sacrificed by decapitation and the hepatic tissues were excised. In case of hepatoma nodulus, a firm white portion was used, after carefully dissecting off the adhering liver tissue and some necrotic material. The tissue was then homogenized with distilled water three times of its weight and subjected to paper electrophoresis. Onto the paper per 1 cm of the initial line 0.01 ml of homogenate was applied as a thin line, under the conditions of 400 volts and the current of 2.5 ma. by using 0.1 M veronal acetate buffer at pH 8.4.

Blood serum. Blood was obtained by cardiac puncture from narcotized animals prior to sacrifice from the above mentioned animals and from which sera were separated. Without dilution 0.01 ml of serum was introduced to the apparatus of paper electrophoresis per 1 cm of the initial line, and the conditions of electrophoresis were entirely the same as those described above.

After 15 hours of electrophoresis the paper strip was removed and was cut into segments in succession in size of 1×1 cm eventually 1×2 cm. Each paper segment was employed as the enzyme source.

Determination of acid phosphatase in hepatic tissues.

The digestion was performed in series of small test tubes each containing 2 ml of solution mixture, which had been prepared by mixing 45 ml of 0.4 per cent disodium p-nitrophenylphosphate, 50 ml of 1 N acetate buffer at pH 4.6 and 5 ml of 0.1 M $MgCl_2$. Paper segment (1×1 cm) was then put into it and incubated at 38°C for 1 hour. The digestion was interrupted by adding 2 ml of 1 N NaOH. Color-developed solution was applied for photo-electric colorimeter using filter 430 mμ.

Determination of alkaline phosphatase in hepatic tissues.

The procedure was similar to the above mentioned determination exept the following points. In a small test tube was transferred 2 ml of solution mixture, which was prepared by mixing 45 ml of 0.4 per cent of the above substrate, 50 ml of 0.1 M barbital buffer at pH 9.4 and 5 ml of 0.1 M $MgCl_2$.

Then the paper segment (1×1 cm) was put into the test tube. After incubation at 38°C for 1 hour, 2 ml of 0.02 N NaOH was pipetted into the test tube to interrupt the digestion. The color thus developed was measured electrophotometrically.

Determination of alkaline phosphatase in sera.

In a series of small test tubes was placed 1 ml of 0.4 per cent solution of substrate and 1 ml of buffer containing $MgCl_2$ at pH 10.5, and the paper segment in size of 1×2 cm was sent to the bottom. The buffer containing $MgCl_2$ was prepared as follows: 7.5 g glycine and 0.095 g $MgCl_2 \cdot 6H_2O$ were dissolved in 700–800 ml water in a volumetric flask of 1-liter capacity and 85 ml of 1 N NaOH was then transferred into it and finally filled up with water to the mark.

The series of test tubes was brought to an incubator at 38°C for 4 hours. Afterwards the digestion was stopped by adding 2 ml of 0.02 N NaOH to each test tube. The color developed was measured electrophotometrically. For the purpose of blank tests untreated filter paper of the same size, which has been soaked with veronal phosphate buffer at 8.4, was treated similary to the above procedure.

The enzyme activity was represented by the amount of p-nitrophenol in γ, which has been liberated from p-nitrophenylphosphate through digestion. The activity was represented graphically to facillitate discussion. On the ordinate the activity was shown by the amount of p-nitrophenol in γ and the abscissa was divided in cm to plot the actual length of electromigration on the paper. When these points were plotted, an activity pattern was ready for discussion.

Results and Discussion

Normal rat liver. The activity pattern of acid phosphatase in the normal liver (Figs. 1-3) showed two prominent peaks fairly separated from each other. The sharp high peak occurred at the initial point and the other lower semi-circular one localized at the migrating zone of protein. Alkaline phosphatase in the normal liver had the low maximum at the starting point and the trace of activity at the migrated part.

Cirrhotic liver. The activity pattern of acid phosphatase in the cirrhotic liver (Figs. 4-6) resembled that of normal liver because of having high level at the starting line and the wide area of activity outlined by semi-circular at the

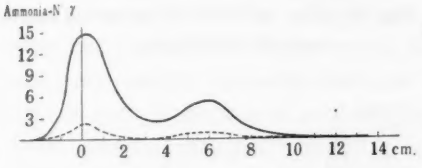


Fig. 1 Normal rat liver

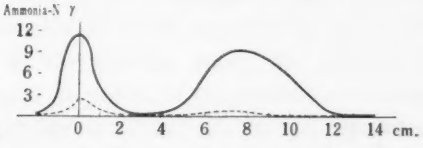


Fig. 2 Normal rat liver

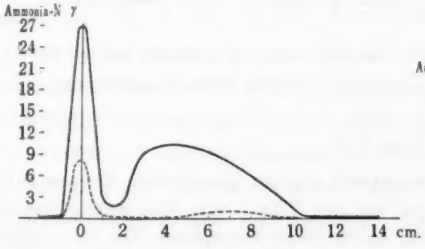


Fig. 3 Normal rat liver

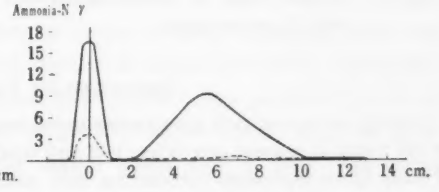


Fig. 4 Cirrhotic liver

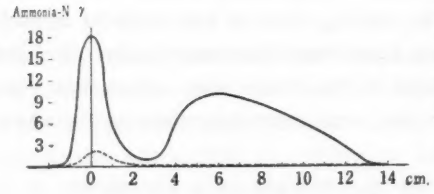


Fig. 5 Cirrhotic liver

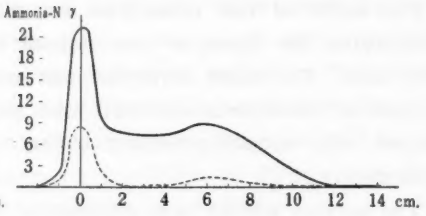


Fig. 6 Cirrhotic liver

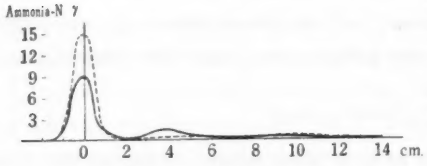


Fig. 7 Hepatoma

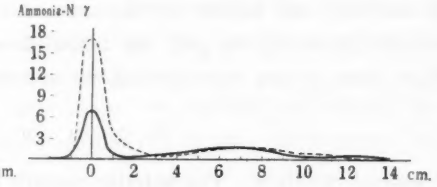


Fig. 8 Hepatoma

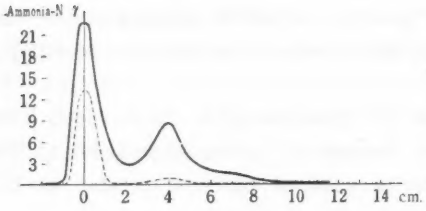


Fig. 9 Hepatoma

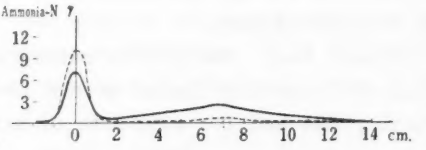


Fig. 10 Liver of DAB fed rat on its fourth week of experiment

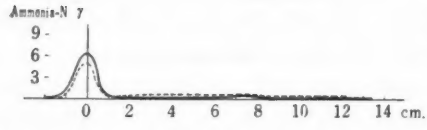


Fig. 11 Liver of DAB fed rat on its fourth week of experiment

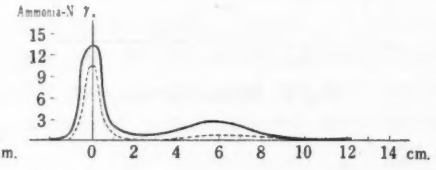


Fig. 12 Liver of DAB fed rat on its fourth week of experiment

Explanation for Figs. 1–12

Activity pattern of acid phosphatase (solid curve) and alkaline phosphatase (broken curue) in the livers of normal rats (Figs. 1–3) and DAB fed rats (Figs. 4–12). The longitudinal line of each figure represents the starting line, onto which tissue homogenate has been applied for paper electrophoresis. The right side of the line is towards the anode.

electromigrated zone. The pattern of alkaline phosphatase activity, on the contrary, showed more marked activity than that of normal liver at the application site. That was conceived as a sign of malignancy, which will be discussed in the case of hepatoma. Thereby it seemed that the pattern of the cirrhotic liver represents a transition from normal to malignant tissue, so far as it concerns the alkaline phosphatase.

Hepatoma. The activity pattern of the acid phosphatase in hepatoma (Figs. 7-8) showed a peak at the application site, but lower than that of alkaline phosphatase, which showed the sharp peak of high level. Both phosphatases were less active at the separated part of protein zone. Thus the pattern of hepatoma phosphatases at the starting point was just the contrary to that of normal liver and cirrhotic liver. In one case of hepatoma (Fig. 9), which was at an early tumor age (nodule weight 0.2 g), showed the pattern resembling that of cirrhotic liver, but was unlike it in high alkaline phosphatase activity at the initial point when observed minutely.

Liver of DAB rats in their fourth week of experiment.

The activity pattern of acid and alkaline phosphatases (Figs. 10-12) resembled those of hepatoma, namely, active alkaline phosphatase at the starting point and low level of acid phosphatase at the same point. Furthermore there were low level of both phosphatases at the separated part of protein. These marked changes in the liver phosphatases, which have taken place in rats already in early experimental days, have not been detected by mere quantitative determination, without using the electrophoretic procedure.

Serum alkaline phosphatase.

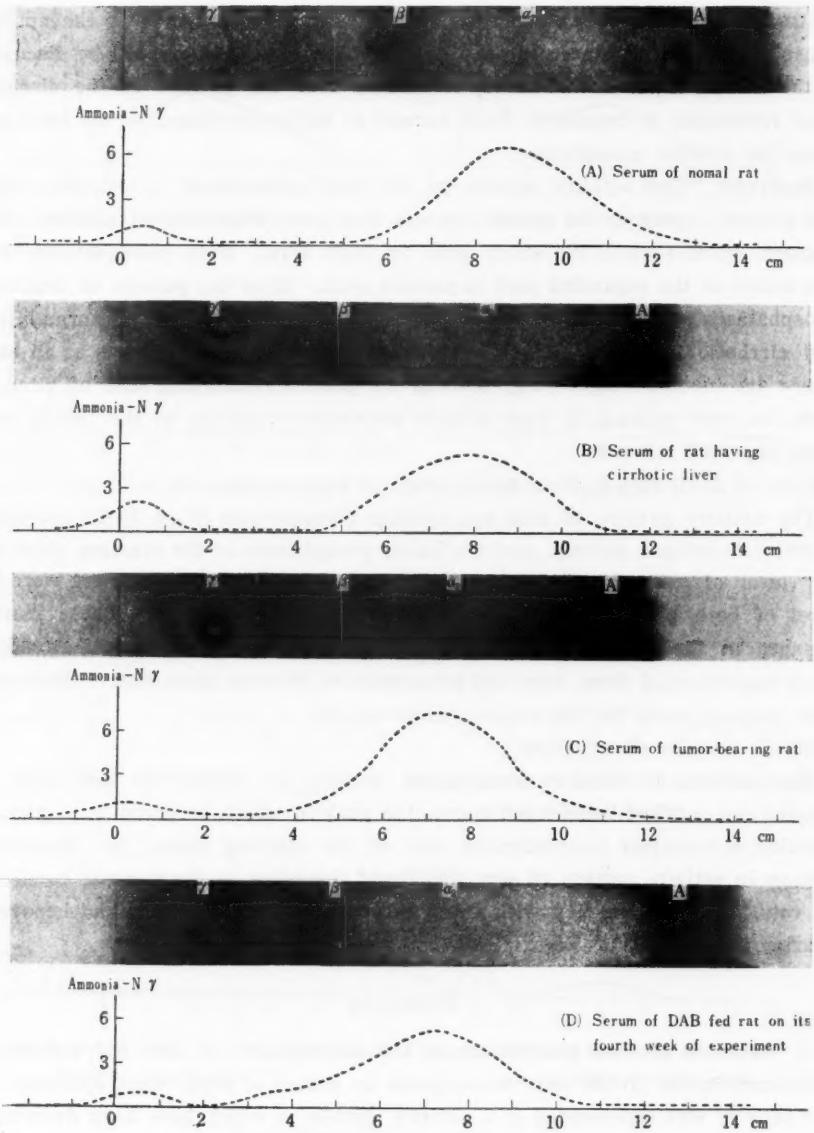
The pattern of alkaline phosphatase activity in normal rat sera (Fig. 13) showed one marked bell-shaped curve, the peak of which occurred in α_2-globulin fraction and another inconsiderable one at the starting point. No remarkable change in activity pattern of sera was found according to the somatic conditions of rats, namely, rats fed with DAB, rats having cirrhotic liver, and hepatoma-bearing rats.

Summary

1) Acid and alkaline phosphatase in the homogenate of rats fed 4-dimethylaminoazobenzene (DAB) were investigated by means of paper electrophoresis and the activity was represented in a pattern, details of which have been described.

2) Pattern of acid phosphatase in normal liver showed two prominent peaks at the initial point and at migrated zone of protein respectively. Alkaline phosphatase showed a low maximum at the initial point and a very low curve situated at the migrated part.

Fig. 13



Explanation for Fig. 13, (A)—(D)

Activity pattern of alkaline phosphatase (broken curve) in blood sera of normal (A) and DAB fed rats (B—D) with the stained strips of corresponding paper electrophoresis. The longitudinal line shows the starting line and its right side is towards the anode.

3) Patterns of both phosphatases in cirrhotic liver were similar to those of normal liver, but there was a higher peak at the initial point, especially in the case of alkaline phosphatase.

4) In case of hepatoma, pattern of alkaline phosphatase showed a peak of high level at the initial point, higher than that of normal liver and even of cirrhotic liver. The peak of acid phosphatase at this site was lower than that of normal and cirrhotic liver.

5) Patterns of both phosphatases in the livers of DAB fed rats in their fourth week were alike to those of hepatoma.

6) Patterns of alkaline phosphatase in sera of DAB fed rats showed non-detectable differences from that of normal rat sera. A marked peak of bell-shaped curve was observed in α_2-globulin fraction and lower one at the starting point.

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要　旨

バターエロー投与シロネズミの肝酵素の濾紙電気泳動的研究
第2報　酸およびアルカリフォスファターゼ
附　血清アルカリフォスファアーゼ

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第1報につづき沪紙電気泳動法によって肝の癌化に伴って起り得る酵素の性格の変化を追究した。ここに従来から癌の酵素の対象としてしばしば取扱われた酸フォスファターゼ (ACP) およびアルカリフォスファターゼ (ALP) を採り上げた。

バターエロー (DAB) 投与ネズミの肝性組織のホモジエネートあるいは血清を沪紙上に電気泳動し，泳動後沪紙を順序正しく多数の小片に切ってそれぞれを酵素源とした。活性度の測定法は一列の小型試験管に基質パラニトロフェニールフォスフェートのヂソヂウム塩溶液とマグネシウムイオンを含む緩衝剤 (酸性およびアルカリ性側) との混合液を分注し，上記沪紙片の一つづつを入れ，一定時間孵卵器中で保温し後強アルカリを加えて産生されたパラニトロフェノールを呈色定量した。この数値をグラフの縦軸に，切り取られた沪紙片の位置すなわち酵素の沪紙上の泳動距離を横軸にとって作成したパターンを論じた。

正常肝 ACP の示すパターンは2つの顕著な山を原点と易泳動部に認めた。前者は鋭い頂点をもち，後者は半円形であった。ALP は原点に低い極大値と易泳動部に極めて低い凸曲線があった。硬変肝 (DAB 長期投与，後正常食で飼育した動物) の ACP, ALP は正常肝のそれぞれに近似しているが，原点の山，とくに ALP のそれは正常肝より著しい。肝癌の ALP は原点に著しく，硬変肝，正常肝より高い。原点の ACP は正常肝，硬変肝より低い。

DAB 投与実験4週の動物の肝は外見正常でありながら ACP, ALP のパターンからはすでに肝癌のそれらに似ていた。血清 ALP のパターンは正常ネズミ血清も DAB 投与ネズミ血清も大差なく，α_2グロブリン分屑に頂点をもつ明瞭な山と原点に位置する低い山を認めた。

(文部省科学研究費による)

[GANN, 49, 295~300; December, 1958]

PAPER ELECTROPHORETIC STUDIES ON ENZYMES IN THE LIVER OF RATS FED 4-DIMETHYLAMINOAZOBENZENE. III. RIBONUCLEIC ACID DESAMINASE

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In our laboratory desaminase activity of ribonucleic acid (RNA) during the development of hepatic tumors on feeding of 4-dimethylaminoazobenzene (DAB) has been studied by Kishi and Haruno. They found two maxima of RNA desaminase in pH curve, situated at pH 5-6 and pH 10-11. RNA desaminase at acid side showed higher activity in the pathological but non-cancerous liver than in normal rat liver. In hepatoma the activity was lowered to the normal level again. RNA desaminase at alkaline side increased its activity stepwise according to the grade of liver lesions, especially marked in hepatoma.

The majority of known enzymes in malignant tissue grew by far less active than those in normal tissue. Among them RNA desaminase in question was considered to be worthy of study from some new points of view, as it seemed to have an unique character.

The present author have undertaken the investigation of RNA desaminase, aiming to study its property using paper electrophoresis.

Materials and Methods

Experimental animals. The rats used in the present experiments were the same as those described in the preceding papers, i.e. DAB rats of prolonged feeding and those in their fourth week of experiment. Untreated rats of the corresponding age and size were employed as the control animals.

The rats were sacrificed and the hepatic tissues were excised quickly and blotted. The hepatic tissue homogenate was prepared with distilled water, exactly three times the weight of tissue, and was used without further treatment (whole homogenate) or fractionated by centrifugation at 15,000 r.p.m. for 1 hour to obtain the supernatant and the sediment. The sediment was diluted with water to the original volume of whole homogenate from which it has been separated.

They were then transferred to an apparatus of paper electrophoresis. The volume of 0.01 ml of the above material was introduced onto the paper 1 cm on the initial line and the current was turned on for 15 hours under the conditions

of 400 volts and 2.5 ma. In the electrode vessels 0.1 M of veronal acetate buffer at pH 8.4 was contained. At the end of this time the strip was taken off from the apparatus and cut into sections in size of 1×1.5 cm. Twenty pieces were thus obtained from the starting line to the anode side and 3 pieces to the cathode side.

Measurement of activity of RNA desaminase.

Desaminase at pH 11. The Conway units, as many as pieces of paper, were employed for ammonia determination.

Into the central well of the units were placed 1 ml of buffer at pH 11 (the mixture of 0.1 M glycine and 0.1 N NaOH) and 1 ml of 0.5 per cent RNA from yeast (E. Merck), which has been adjusted to the same pH by NaOH. The paper segment (1×1.5 cm), as a source of the enzyme, and some toluene were also transferred into the well. The above mentioned procedure was of course an unusual use for the central well, but it was advantageous to the uniform elution of the enzyme from the paper into the mixture, without needing to cut paper into smaller pieces. Into the outer chamber of the units 2 ml of 0.02 N H_2SO_4 was placed this time. Then the greased lid of the unit was fixed in proper position and incubated at 38°C for 22 hours. In the meantime, the liberated ammonia was trapped in the H_2SO_4. After the time is up, 2 ml of Nessler's reagent was added and the nesslerized solution was briefly centrifuged. The amount of ammonia was determined by a photo-electric colorimeter.

Desaminase at pH 5.8. The procedure was similar in major part to the above mentioned determination at pH 11. The used buffer was phosphate mixture at pH 5.8 and RNA solution at the same pH as substrate. Incubation time of the units was 20 hours. Then the lids of the units were cautiously opened and 2 drops of saturated K_2CO_3 solution were added to the digestion mixture in order to alkalize it. Then the lids were refixed immediately and the units were placed in an incubator at 38°C and the distillation of ammonia was allowed to proceed for 2 hours. Further procedure was the same as in case of determination at pH 11.

The remaining paper strip was stained by bromophenol blue to see the distribution pattern of protein zones. In respect of this, the stained paper strip was at first cut into segments (0.5×0.5 cm) and the amount of dye was measured respectively by photoelectric colorimeter after the elution of dye has been accomplished by 5 ml of 0.01 N NaOH.

Results and Discussion

It was advantageous to represent the activity of RNA desaminase in a pattern. In abscissa nitrogen γ of liberated ammonia through digestion was taken and in ordinate actual distance of electromigration on the paper in cm, onto which hepatic tissue homogenate had been introduced for electrophoresis at the starting line.

When the points were plotted an activity pattern was obtained.

All the patterns of whole homogenate at pH 11 and pH 8.5 were akin to each other, notwithstanding that they were obtained from the liver of rats in different conditions, i.e., normal rat liver (Fig. 1, Fig. 6), cirrhotic liver (Fig. 2, Fig. 7), hepatoma (Fig. 3, Fig. 8), the liver from rats in their early experimental days (Fig. 4, Fig. 9), and the regenerating liver (Fig. 5).

At the initial point the pattern showed a peak and decreased once a little, then passed over to a plateau, which continued within the whole boundary of protein zone, then fell promptly to zero.

It was obvious from the above results that the RNA desaminase distributed widely among all sorts of hepatic tissue protein. So the protein portion of the above enzyme was not simple. Futhermore, it might be suggested that the RNA desaminase was not affected qualitatively by pathological changes of the liver in the course of tumor development. The pattern of supernatant represented the fast moving part of whole homogenate (Figs. 1A–4A). The patterns of sediment showed that the moving was retarded, representing the immovable part of whole homogenate (Figs. 1B–4B). Thus the supernatant and the sediment each expressed a part of the pattern of whole homogenate. In other words, if the pattern of supernatant was superposed on that of sediment it may reproduce the pattern of whole homogenate.

Activity patterns of RNA desaminase at pH 11

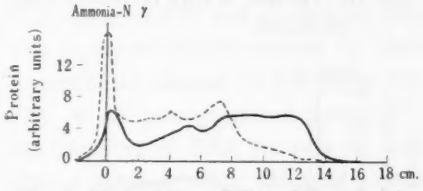

Fig. 1 Normal liver (Whole homogenate)

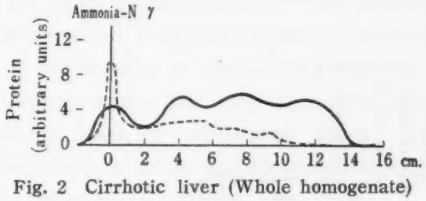

Fig. 2 Cirrhotic liver (Whole homogenate)

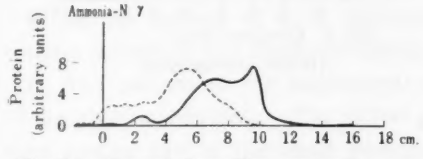

Fig. 1A Normal liver (Supernatant)

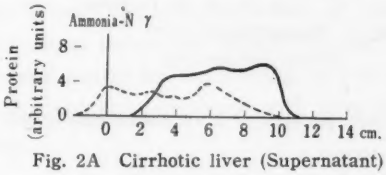

Fig. 2A Cirrhotic liver (Supernatant)

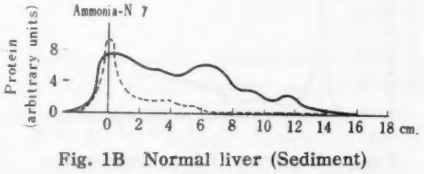

Fig. 1B Normal liver (Sediment)

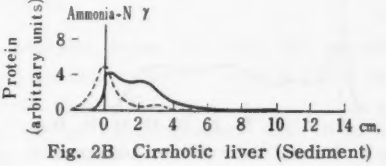

Fig. 2B Cirrhotic liver (Sediment)

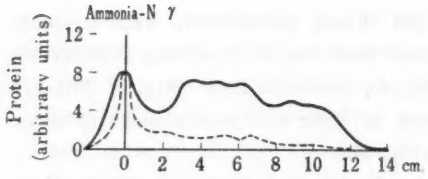

Fig. 3 Hepatoma (Whole homogenate)

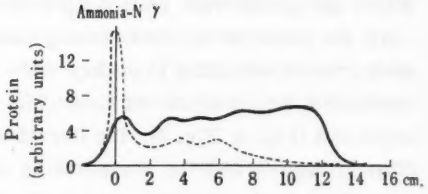

Fig. 4 The liver of DAB rat (fourth week) (Whole homogenate)

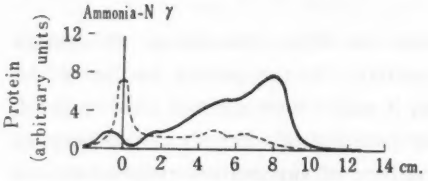

Fig. 3A Hepatoma (Supernatant)

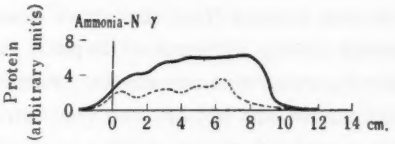

Fig. 4A The liver of DAB rat (fourth week) (Supernatant)

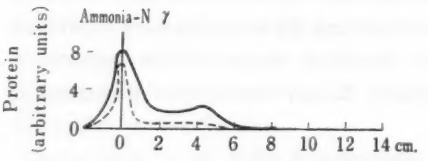

Fig. 3B Hepatoma (Sediment)

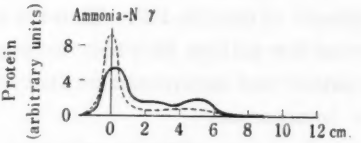

Fig. 4B The liver of DAB rat (fourth week) (Sediment)

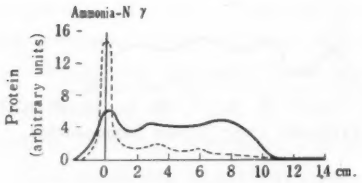

Fig. 5 Regenerating liver* (Whole homogenate)

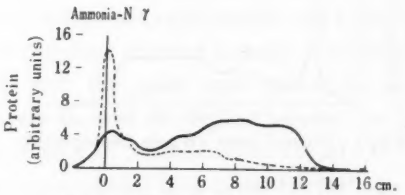

Fig. 7 Cirrhotic liver (Whole homogenate)

Activity patterns of RNA desaminase at pH 5.8

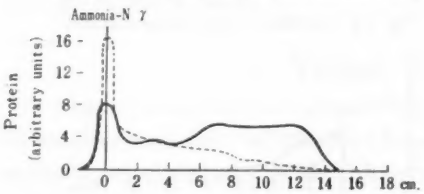

Fig. 6 Normal liver (Whole homogenate)

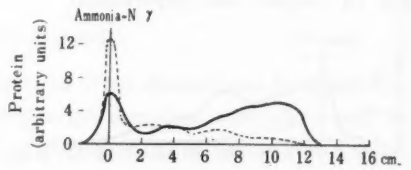

Fig. 8 Hepatoma (Whole homogenate)

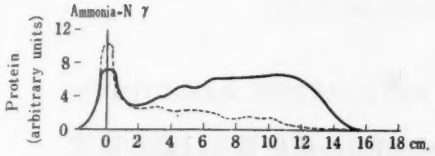

Fig. 9 The liver of DAB rat (fourth week)
(Whole homogenate)

Explanation for Figs. 1-9

Patterns of RNA desaminase activity at pH 11 (solid curve) of the whole homogenate of hepatic tissues are shown in Figs. 1-5, those of the supernatant in Figs. 1A-4A, and those of the sediment in Figs. 1 B-4 B.

Patterns of RNA desaminase activity at pH 5.8 (solid curve) of the whole homogenate of the hepatic tissues are shown in Figs. 6-9.

Distribution pattern of hepatic tissues protein on the strips of the paper electrophoresis of all corresponding measurements are represented in broken curves.

Longitudinal line of each figure shows the starting line of the paper electrophoresis and its right side is towards the anode.

*) The liver was obtained from the rat, which had been partially hepatectomized twice successively with an interval of 48 hours and sacrificed at 48 hours after the second operation.

Summary

1) Ribonucleic acid (RNA) desaminase in the liver of rats fed 4-dimethylaminoazobenzene (DAB) was studied by means of paper electrophoresis. The activity was expressed by the amount of liberated ammonia and the extent of electromigration of enzyme on the paper was also represented on the activity pattern.

2) Whole homogenate of normal liver, the pathological liver including hepatoma, and the liver of DAB fed rats in their fourth week of experiment showed the similar pattern, in the shape of plateau extending to whole protein zone, although having a slight undulation especially at the peak of the starting line.

3) In the pattern of RNA desaminase in acid and alkaline media, no characteristic difference was observed among the above mentioned hepatic tissues.

4) The pattern of the supernatant covered nearly all the moving part of that of the whole homogenate. The pattern of the sediment coincided with that of the slow moving part of the whole homogenate.

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要　旨

バターエロー投与シロネズミの肝酵素の濾紙電気泳動的研究
第3報　リボ核酸脱アミノ酵素

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(昭和医科大学生化学教室，指導　岸　三二教授)

バターエロー (DAB) 投与ネズミの肝癌生成過程において予想し得る肝酵素の性格上の相違を追究することを目的とした。対象とした酵素は当研究室でかつて調べたリボ核酸 (RNA) 脱アミノ酵素である。しかし従来の単なる活性度の定量でなく，性格を調べるため沪紙電気泳動法を用いた。

肝性組織のホモジェネートあるいはその上清，沈澱を沪紙上に電気泳動し，泳動後沪紙を多数の小片に切りそれぞれを酵素源とした。

活性度の測定にはコーンウェイ装置を使用し，基質 RNA と緩衝剤 (酸性側およびアルカリ性側) の混合液から上記沪紙片によって一定時間保温，産生されたアンモニアを装置の硫酸部に捕捉しネスレル化して比色定量した。グラフの縦軸にアンモニア量を，横軸に酵素の泳動距離を目盛って RNA 脱アミノ酵素 (酸性側およびアルカリ性側) のパターンを作成して論じた。

肝性組織 (正常肝，硬変肝，肝癌，DAB 投与4週動物の肝) のホモジェネートは総て共通のパターンで台地形曲線を示した。すなわちいづれも展開された蛋白部全般に亘ってほぼ同程度の活性度であった。ただ原点に山形曲線が現われた。

ホモジェネートの上清および沈澱のそれぞれのパターンはホモジェネートそのままが示したパターンの各一部づつを現わした。すなわち上清は易泳動部を沈澱は難泳動部 (原点およびその隣接部) を示した。

(文部省科学研究費による)

[GANN, 49, 301~306; December, 1958]

TRYPTOPHAN METABOLISM IN TUMOR-BEARING ANIMALS

KAZUO MORI, SHOGO ICHII and KATSUHIKO MATSUMOTO
(Department of Medical Zoology, Showa Medical School, Tokyo)

Recently the recognition of abnormalities in tryptophan metabolism in many diseases has aroused a great interest in clinical and experimental investigations. The present communication deals with studies on the urinary excretions of tryptophan, kynurenine and anthranilic acid in tumor-bearing animals before and after administration of tryptophan.

Material and Method

Three groups of subjects were studied: (1) rats during the course of hepatic carcinogenesis by azo-dye feeding, (2) rats bearing transplantable hepatoma (AH 49), and (3) mice bearing Ehrlich ascites carcinoma.

(1) Male Wistar rats were fed p-dimethylaminoazobenzene so as to produce liver cancer routinely. Five rats of the group were examined at one month after the beginning of the feeding. The rest of animals was continued on diet containing p-dimethylaminoazobenzene at a level of 0.06 per cent for 4 months and then returned to a basal diet without dye for an additional one month. After that, each animal was housed in a screen-bottomed metabolism cage and fed bread, cabbage and water freely. And the 24 hour urine was pooled. At second experimental day, each rat received tryptophan in amount of 100 mg per 100g body weight orally in a single dose. And the next 24 hour urine was collected also. Colorimetric assay on urinary excretions of tryptophan, kynurenine and anthranilic acid in rats were made before and after administration of tryptophan. For comparison, similar studies were made on normal animals.

Tryptophan, kynurenine and anthranilic acid with unknown diazotizable substances of metabolites were determined by the method of Eckert (1) which was followed by Mason and Berg (2). Since kynurenine and anthranilic acid also respond by diazotization with sodium nitrite, the optical density attributable to tryptophan alone was substructed under this procedure.

Prior to analysis, 7 ml of distilled water and 2 ml of trichloroacetic acid (25 per cent) were added in turn to 1 ml of the urine and then the mixture was centrifuged. Four ml of 5 per cent of trichloroacetic acid was added to 1 ml of the supernatant. And four tubes of the samle for assay were prepared simultaneously. Three tubes of the sample were for diazotization and the remaining one was for blank test.

After development of the color by the addition of N-(1-naphthyl) ethylenediamine, the optical density at 550 mμ was measured in a Hitachi's photoelectric photometer, and compared with standard curve prepared previously.

After the estimation of the urinary excretions of different metabolites for two consecutive days, animals were killed by exanguination and livers were classified into two grades pathologically, cirrhosis and liver cancer.

(2) Male Wistar rats were given the transplantable hepatoma (AH 49) subcutaneously on the back by the syringe methods. This strain of AH 49 is an ascitic carcinoma essentially. Approximately two weeks after transplantation, when the tumor mass was 10–15 per cent of the body weight, the animals were housed singly, in screen-bottomed metabolism cage with free access to bread and water. Urinary excretion of tryptophan metabolites were determined on two consecutive days, before and after the administration of tryptophan, by the same method as described above.

(3) Female adult mice of dd strain, weighing 18 g, were used in this experiment. Mice received the inoculation of Ehrlich ascites carcinoma subcutaneously and then allowed to grow the solid tumor. One or two weeks later, each five mice was housed together in screen-bottomed metabolism cage and fed bread, cabbage and water freely. The 24 hour urine was pooled. At second experimental day

Table 1. The 24 hours urinary excretion of tryptophan and its metabolites after tryptophan loading.

Animals	No. of rats	Tryptophan loading	Tryptophan T (mg)	Kynurenine K (mg)	Anthranilic acid (mg)*	K/T
Normal	10	Before	2.14 (1.59–2.80)	0.44 (0.19–0.68)	0.87 (0.31–1.36)	0.206
		After	14.32 (6.18–34.05)	3.60 (0.84–8.14)	2.53 (1.05–5.20)	
		Increment	12.18	3.16	1.71	
Rats fed azo-dye for one month	5	Before	2.38 (1.55–3.15)	0.53 (0.32–0.88)	0.67 (0.58–0.79)	0.222
		After	22.38 (18.9–30.3)	10.50 (5.36–16.00)	8.63 (5.44–10.70)	
		Increment	20.00	9.97	7.96	
Rats with cirrhotic liver	8	Before	2.04 (0.90–2.90)	0.19 (0.00–0.50)	0.91 (0.43–1.41)	0.093
		After	6.59 (2.85–10.8)	1.45 (0.32–3.30)	1.91 (0.98–3.84)	
		Increment	4.43	1.26	1.00	
Rats with liver cirrhosis and cancer**	9	Before	2.08 (0.99–4.01)	0.16 (0.00–0.52)	0.74 (0.57–1.02)	0.077
		After	7.23 (2.36–15.65)	0.93 (0.13–1.73)	1.87 (0.74–3.14)	
		Increment	5.15	0.77	1.15	

* Contained unknown diazotizable metabolites.

** The weight of liver cancer was about 10 per cent of body weight of the host.

mice received intraperitoneal injection of tryptophan in amount of 100 mg per 100 g of body weight in a single dose. And the colorimetric assay on urinary excretions of tryptophan, kynurenine and anthranilic acid with unknown diazotizable substances was carried out before and after tryptophan loading. Similar studies were made on normal mice also.

Results

(1) The results obtained in rats during the course of cancer production are shown in Table 1. The increment in excretion of tryptophan and its metabolites attributed to the administration of tryptophan is calculated as the difference between the basal excretion and the excretion in 24 hours following the administration of tryptophan.

In the rats fed azo-dye for a month, the excretion of tryptophan and its metabolites is greater than normal, before and after tryptophan loading. And the ratio of kynurenine to tryptophan levels was 0.222, while it was 0.206 in normal. Between the normal and experimental rats with cirrhotic or cancerous livers, no remarkable difference was observed in the basal excretion for tryptophan. After the loading of tryptophan, however, the excretion of tryptophan became less than half of normal in the urine of experimental animals. As for kynurenine, the level both in basal and after the tryptophan loading was less than half of normal in the experimental animals. Then the ratio of kynurenine to tryptophan levels were 0.075 or 0.077 in rats with cirrhotic or cancer livers respectively. On the other hand, the amounts of anthranilic acid and unknown diazotizable substances in the urine of rats in normal and experimental groups were found almost in the same range both before and after tryptophan loading.

Table 2. The 24 hour urinary excretion of tryptophan and its metabolites following the administration of tryptophan in rats bearing transplantable hepatoma (AH 49).

Treatment	No. of Rats	Tryptophan T (mg)	Kynurenine K (mg)	Anthranilic acid (mg)	K/T
Before	10	1.09 (0.73–1.46)	0.20 (0.13–0.34)	0.63 (0.12–0.25)	0.18
After		5.34 (3.09–13.90)	1.70 (1.31–3.50)	0.78 (0.60–1.17)	
Increment		4.25	1.50	0.15	

(2) The values obtained in the tumor bearing rats are presented in Table 2. As described above, the estimations were made two weeks after the tumor inoculation. From the table it is seen that tryptophan and its metabolites are less than the normal. All values presented more resemble those in the urine of

rats with cirrhotic liver than of other rats. And the ratio of kynurenine to tryptophan was 0.18 in this subject.

(3) The results obtained in mice bearing Ehrlich ascites carcinoma are shown in Table 3. It is seen that the basal excretion of tryptophan and anthranilic acid in the urine of tumor bearing mice became less than normal. Moreover in the tumor bearing mice the increase in excretion of tryptophan and anthranilic acid following tryptophan loading was not remarkable when compared with normal. While the basal urinary excretion of kynurenine, which was absent initially, appeared in the tumor bearing mice. The ratios of kynurenine level to tryptophan in mice with Ehrlich tumor were 0.04 or 0.14 after one or two weeks respectively following the transplantation.

Table 3. The 24 hour urinary excretion of tryptophan and its metabolites after the the adiministration of tryptophan in mice bearing Ehrlich ascites tumor.

Days after transplantation	No. of mice	Treatment	Tryptophan T (mg)	Kynurenine K (mg)	Anthranilic acid (mg)	K/T
0	40	Before	0.42 (0.39–0.50)	0 (0.00–0.00)	0.09 (0.40–0.14)	0
		After	2.26 (1.46–3.06)	0.34 (0.22–0.58)	0.12 (0.07–0.22)	
		Increment	1.84	0.34	0.03	
7	15	Before	0.23 (0.21–0.26)	0.01 (0.01–0.01)	0.04 (0.03–0.08)	0.04
		After	2.82 (1.64–4.54)	0.39 (0.22–0.50)	0.06 (0.04–0.08)	
		Increment	1.59	0.38	0.02	
14	20	Before	0.21 (0.19–0.20)	0.03 (0.02–0.04)	0.04 (0.03–0.05)	0.14
		After	1.41 (1.33–1.52)	0.24 (0.16–0.43)	0.03 (0.01–0.06)	
		Increment	1.20	0.21		

Discussion

Following oral administration of tryptophan to diseased subjects the urinary excretion of various tryptophan metabolites was found to be decreased (7), and this abnormality was also observed in diabetic rats (3). Moreover, the abnormalities in tryptophan metabolism in tuberculosis (5), cancer (6), malignant carcinoid, liver cirrhosis, diabetes and infective hepatitis (7) are becoming importantly recognized in the clinic lately. In the experiments described in this paper it was found that in the precancerous conditions in azo-dye fed rats urinary excretion of tryptophan was less than normal before and after the feeding of tryptophan. And these decrease in urinary excretion of tryptophan were observed in the tumor bearing mice.

The first step in tryptophan metabolism in vivo is presumably oxidation by tryptophan peroxidase (TPO), and subsequently kynurenine is formed. TPO activity in tumor-bearing mice was found to be significantly higher than that of normal (8, 9). It is reasonable to consider that the appearance of kynurenine in urine in tumor-bearing mice was caused by the accelerated activity of TPO in these animals. Furthermore, TPO activity increased adaptively after the administration of tryptophan to animals. The degree of adaptive increment of TPO activity has been found to be diminished in mice during the tumor growth. So, the fact that the increment of kynurenine after the administration of tryptophan in mice with advanced tumor was less than that of normal may be due to the diminished adaptability of TPO in tumor-bearing mice.

In rats, TPO activity in cirrhotic liver was decreased to 2/3 the level of normal and in liver cancer the amount of kynurenine formed was found to be negligible. The decreased urinary excretion of kynurenine in rats during hepatic carcinogenesis may be comparable to the activity of TPO in pathological liver tissues.

Kynurenine formed by the action of TPO is further metabolized by the enzyme contained in the liver and kidney of rats (2), and this enzyme is present in higher concentration in the kidney than in the liver. The urinary kynurenine will be the residual one that dose not metabolized in either liver or kidney in rats. The amount of kynurenine in urine seems to be affected by not only by liver function but also by the renal function.

Anthranilic acid is the further metabolite of kynurenine. In the rat, the anthranilic acid was increased markedly after the tryptophan loading but the increment of anthranilic acid after loading is only slight in mice. This suggests that there is a difference in the pathway of kynureinine metabolism between mice and rats. The metabolic difference between rats and mice is already mentioned by Brown and Price (10).

Summary

The present communication deals with studies of the urinary excretions of tryptophan, kynurenine and anthranilic acid in tumor bearing rats or mice before and after the administration of tryptophan.

In the rats with cirrhotic or cancerous livers urinary excretion of tryptophan and its metabolites were less than normal. And the urinary excretion of tryptophan in tumor-bearing mice was of subnormal proportion. However, the kynurenine level in the urine was high in these mice.

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要　旨

担癌動物のトリプトファン代謝

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1) p-Dimethylaminoazobenzene 肝癌生成過程の白鼠，腹水肝癌移植白鼠あるいはエーリッヒ癌移植廿日鼠尿中のトリプトファン，キヌレニン並びにアントラニール酸等を，トリプトファン負荷前後に比色定量して，それらの動物のトリプトファン代謝を検討してみた。

2) 肝硬変あるいは肝癌をもった白鼠の尿中のトリプトファン並びにその代謝物は，いづれも正常白鼠尿中のそれらより低い値を示した。尚動物にトリプトファンを負荷した後のトリプトファン並びにその代謝物の増量も軽度であった。

3) 腹水肝癌移植白鼠でも，トリプトファン並びにその代謝物の尿中えの排泄は正常鼠に比べて低値を示していた。

4) エーリッヒ癌廿日鼠では，尿中えのトリプトファン並びにアントラニール酸の排泄量が正常の場合より減じている。しかしキヌレニンの尿中えの排泄量は癌移植後の発育につれて増してくることがわかった。

5) 以上の結果から白鼠と廿日鼠では，癌のある場合のトリプトファン代謝過程がちがうようにおもわれる。

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EFFECTS OF X-RAY IRRADIATION ON THE TRANSMISSIBLE VENEREAL TUMOR OF THE DOG

(Plates XXII—XXV)

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INTRODUCTION

The transmissible venereal tumor of the dog is a neoplasm developing in the external genitals of both male and female dogs. It has been encountered in approximately three percent of dogs seen in the University of Tokyo Veterinary Hospital. In Japan it is usually called "polyp" because of its gross appearance whereas in the United States and European countries a variety of names has been applied including Sticker's sarcoma, venereal granuloma or contagious lymphosarcoma. Although the etiology has not been fully clarified, it is generally accepted that the tumor is transmitted between sexes by copulation (Smith 1898, White 1902, Watanabe 1956).

Pathologically the tumor has usually been classified as a round cell sarcoma (Smith 1898, Sticker 1906, Yamagiwa 1908, Matsui 1909, Imamaki 1932, Watanabe 1956). Other pathologists have considered it to be an endothelioma (Beebe 1906), a neuroblastoma (Jackson 1944), a histiocytoma (Mulligan 1948), or a reticular monocytoma (Nanta 1949). The tumor appears to be malignant on the basis of its histological characteristics since abundant mitoses are ordinarily observed. Furthermore, metastasis may occur to the inguinal lymph nodes or, less commonly, to the spleen, liver, lung, or subcutaneous tissues. Rarely, death ensues as the result of widespread metastases, uremia due to urethral obstruction, or septicemia following necrosis and secondary infection.

The tumor is carried by the great majority of dogs for long periods, up to several years, without serious deleterious effects on the general condition of the animals. In some instances, the tumor appears to shrink and disappear spontaneously. Consequently, the malignancy of this tumor is regarded by most authors to be of relatively low degree.

Using viable cell suspensions experimental transplantation from dog to dog, either by scarification of the genital mucosa or by subcutaneous implantation, has been accomplished frequently (Wehr 1889, Smith 1898, Sticker 1904 and 1906, Wade 1908, Matsui 1909, Matsuba 1927, Imamaki 1932, Stubbs 1934, Tsuchie 1941,

Thiery 1950). Further, experimental passages of the tumor through many generations have been reported (Karlson 1952). The tumor has never been transplanted by cell-free materials. However, attempts to demonstrate filtrable causative agents have been unsuccessful (Sticker 1906, Stubbs 1934, De Monbreun 1934). Nevertheless, the possibility of a viral origin must be considered (Nanta 1951).

In therapy, surgical excision or cauterization has been generally employed but incomplete removal frequently results in recurrence. Recently, treatment by x-ray irradiation has been reported. Banks (1954) described a single case treated successfully with a total dose of 2244r given in seven exposures over a twenty-two day period. Marquès et al (1952) reported a high recovery rate in cases treated by irradiation but no details were given.

This paper describes the results of x-ray irradiation therapy in eight dogs. Histologic changes produced by treatment were followed by serial biopsies in all animals. In five dogs, permitted to survive, follow-up observations have been carried out for two years or more.

Material and Methods

Eight female dogs with spontaneous single or multiple transmissible venereal tumors of the vagina, of one to twelve month duration, were used in these studies. In each case, a part of the tumor protruded between the labia so that observation and biopsy were convenient.

X-ray treatments were administered twice a week over a period of three weeks. Total dosage ranged from 1200r to 2400r with six exposures of 200r in two dogs (No. 1 and 2), or 300r in four dogs (No. 3, 4, 5 and 6) and 400r in two dogs (No. 7 and 8). The quality of radiation employed was as follows: 150 KVp, 3 mA., filter 0.5 mm. Cu. plus 1.0 mm. Al, anode-skin distance 30 cm., field 4×6, 6×8, or 8×10cm. corresponding to the size of tumor, dose rate 10.56 to 16r per minute. The dogs were irradiated under general anesthesia with thiopental sodium.

Biopsies were made a few days before, immediately before, immediately after, and one, three, six, twelve, twenty-four and forty-eight hours after the first irradiation treatment. Thereafter, biopsies were made at variable intervals following therapeutic exposures until the tumor disappeared. Tissues taken for histological study were fixed in Bouin's solution and ten percent formalin, and sections were stained with hematoxylin-eosin, Heidenhain's iron hexatoxylin and Heidenhain's azan. For fat staining, frozen sections were stained with Sudan III.

Hematological studies were also performed during and following treatment. Hemoglobin contents were determined by the oxy-hemoglobin method. Total white blood count using four counting chambers was carried out in the usual manner.

Blood smears were stained with Wright's stain and 400 leukocytes were counted.

Results

A. Gross Changes in the Tumor

Observations before irradiation (Table 1, Fig. 1, Fig. 5):

In some cases (No. 1, 2 and 3), one or more tumors involved only the vaginal wall adjacent to the labia whereas in others (No. 4, 5, 7 and 8) massive tumors spread widely over the entire vaginal wall almost occluding the vulvo-vaginal lumen and elevating the skin over the perineum. Some tumors were pedunculated, others were attached by a broad base to the vaginal wall. The pink or red surface was finely granular. Consistency varied from soft and fragile to firm. They bled easily when handled and had a characteristic disagreeable odor.

Observations after irradiation (Fig. 2, Fig. 3, Fig. 6):

The surface of the tumors was covered by a seromucous discharge six to forty-eight hours after the first irradiation, and the granular appearance gradually became smooth during the period of the treatment. Reduction in size and decolorization became evident following the third or fourth treatment. Thereafter, the tumors regressed rapidly. In one dog (No. 2) the tumor disappeared completely three days after the fourth irradiation treatment, whereas in a less sensitive case (No. 8) complete regression of the tumor did not occur until one month after the sixth treatment. After six exposures (1200 to 2400 r), complete disappearance of the tumors was observed (Fig. 3, Fig. 6) in all cases except one (No. 5). In the latter animal, the superficial masses adjacent to the vulva disappeared following the final x-ray exposure, but the autopsy revealed surviving tumor tissue located deep in the vaginal lumen adjacent to the cervix (Fig. 4).

Follow-up observations on the results of irradiation have been made for more than two years. No relapse of the tumor has occurred in five surviving dogs. In addition, two dogs have undergone successful pregnancies with normal parturition (Table 1).

B. Histologic Changes in the Tumor

Observation before irradiation (Fig. 7, Fig. 8 and Fig. 9):

In the tumor tissues from eight dogs examined before irradiation, parenchymal tumor cells were round, oval or polyhedral and displayed a striking uniformity in size and shape. Cell boundaries were clearly revealed with either the azan or iron hematoxylin stains; with the hematoxylin-eosin stain cell borders were less distinct. The nuclei were large, round or oval in shape, and included clumps of fine chromatin together with a single prominent nucleolus. Mitoses in various stages were frequently observed.

Micrometer measurements revealed diameters of cells, nuclei and nucleoli ranging

Table 1. The dogs with transmissible venereal tumors treated by x-ray irradation.

Dog No.	Bread	Sex	Age years	Duration after detection of growth	Location and size of the tumor	Observations after recovery and other remarks
No. 1	Mongrel	f.	2	1 year	Vaginal vestibule 5×7×1.5 cm.	No sign of recurrence in more than two years.
Na. 2	Japanese Akita	f.	3	7months	All over the vaginal wall near the vulva 7.5×6×1.5 cm.	No sign of recurrence in more than two years.
No. 3	Japanese Shiba	f.	4	1 month	Left vaginal wall near the vulva. 3×4×2 cm.	No sign of recurrence in more than two years.
No. 4	Mongrel	f.	6	1 year	All over the vaginal wall. 5×7×4 cm., measured over the skin	Destroyed five months after recovery. No vestige of the tumor.
No. 5	Mongrel	f.	Uncertain		All over the vaginal wall, 1 to 2 cm. thick.	A part of the tumor remained at autopsy 32 days after the end of the treatment.
No. 6	Mongrel	f.	Uncertain		A pigeon egg size tumor at the ventral vaginal wall.	Parturition two times, died two years after recovery. No vestige of the tumor.
No. 7	Mongrel	f.	4	6 months	All over the vaginal wall, and the vagina was full of the growth. 13×7×6 cm. measured over the skin.	Destroyed one month after the end of the treatment. No tumor tissue.
No. 8	Mongrel	f.	6	1 year	All over the vaginal wall and the vagina was full of the growth. 10×5×5 cm. measured over the skin.	Parturition one time. Destroyed two years after the end of the treatment. No. vestige of the tumor.

from 8 to 12μ, 6 to 7μ and 1 to 2μ, respectively. The cells also contained considerable cytoplasm which stained light pink with eosin. Small lipid droplets, varying widely in size and number, were demonstrated in the cytoplasm with Sudan III stain. A small number of karyolytic or pyknotic cells was also observed. The fibrous stroma was usually scant but was increased in some cases. The azan stain clearly showed a small number of collagenous and reticulum fibers among the parenchymal cells (Fig. 8). Small numbers of lymphocytes and granulocytes were scattered throughout the stroma.

Observations after irradiation:

Within three to six hours after the first irradiation, mitoses of the tumor cells almost entirely disappeared in all cases. A few abnormal mitotic figures with chromosome bridge formation, acentric chromosomes, or scattered chromosomes

occasionally observed during this period (Fig. 19). After this temporary suppression, mitosis reappeared in the tissues biopsied at twelve hours after the first irradiation, but some of these mitotic figures showed abnormality. The number of abnormal mitotic figures was gradually increased in course of time after the first irradiation. The abnormal mitoses observed in the tissues biopsied twelve to forty-eight hours after the first irradiation represented, in the main, deviations from the stage of prophase or metaphase; less commonly, abnormalities of the anaphase or telophase were seen. Sticky chromosomes, chromosome bridges, asymmetrical division, scattered chromosome fragments or acentric chromosomes were the aberrations observed in this period (Fig. 10, Fig. 20, Fig. 21).

In one case (No. 8), perivascular cuffs of tumor cells were observed in the material biopsied forty-eight hours after the first irradiation. Tumor cells lying remote from the capillary vessels showed karyolysis or pyknosis, whereas the tumor cells in the layer near the capillary vessels were relatively unaffected (Fig. 16).

Enlargement of both the tumor cells and their nuclei was generally observed in the tissues biopsied twenty-four hours after the first irradiation; this change was less noticeable in earlier stages (twelve hours), but more distinct in later biopsies (forty-eight hours). However, after the second exposure, cellular and nuclear enlargement became more marked; the diameters of the cells and the nuclei increased to 13 to 15μ and about 10μ, respectively (Fig. 11, Fig. 12). In this stage of degeneration, cellular and nuclear disintegrations including deformity of the cell membrane, vacuolization, loosened chromatin network, marginal hyperchromatosis, karyolysis, and pyknosis were observed in almost all cells (Fig. 11, Fig. 12, Fig. 13). In addition, most of the mitotic figures still observed in the disorganized tissues were abnormal, with scattering of chromosomal materials in the enormously enlarged nuclei being predominant (Fig. 23).

There were no discernible changes in the stroma observed for a few days following the first irradiation. However, the stromal response became more conspicuous after the second irradiation (six to nine days after the first irradiation). In this stage of tumor degeneration, the nuclei of fibrocytes and endothelial cells of capillary vessels became round and the nucleoli of these cells were clearly visible with azan stain. In parallel with these changes, fibroblastic proliferation was observed (Fig. 12, Fig. 14).

Degeneration of tumor cells became remarkably intense after the third or fourth irradiation (nine to thirteen days after the first irradiation) and many granulocytes, histiocytes, lymphoid cells and plasma cells appeared (Fig. 12, Fig. 24).

In the final stage of tumor regression, after the fifth to sixth irradiation (fourteen to twenty days after the first irradiation), conspicuous proliferation of fibrous tissue (stained bluish with azan stain) was observed (Fig. 15, Fig. 18). In this

fibrous tissue a few degenerating tumor cells were found scattered with infiltration cells.

C. Hematological Observations

Before irradiation all dogs showed a leukocytosis, predominantly neutrophilic, up to 20,000 cells per cubic millimeter. By the time treatment was completed the white-cell count returned to normal. However, prior to the decrease in white-cell count, a transient increase developed in four cases a few days after the first irradiation. A slight fall in eosinophils was observed in five cases within ten days following the first irradiation. Lymphocytes and monocytes showed no definite or consistent changes in number. Both the hemoglobin content and hematocrit decreased slightly in some cases during treatment. Typical blood changes, observed in two cases, are shown in Charts 1 and 2.

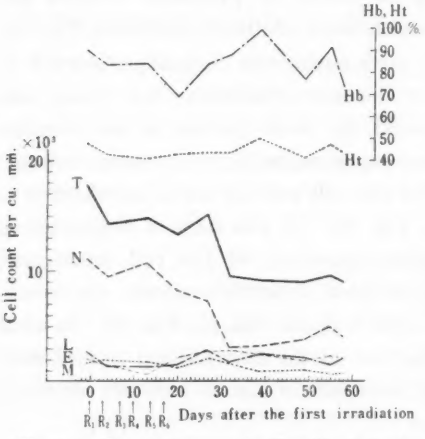

Chart 1 Blood changes in Dog No. 1 during and after the radiation taeatment.

Hb. Hemoglobin content, Ht. Hematocrit, T. Total white cell counts, N. Neutrophiles, L. Lymphocytes, E. Eosinophils, M. Monocytes, R. Irradiation.

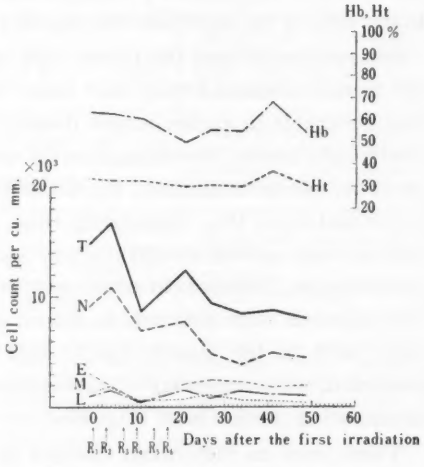

Chart 2 Blood changes in Dog No. 8 during and after the radiation treatment. Legend shown on Chart 1.

Discussion

A temporary inhibition of cell division seems to be a characteristic effect of radiation, having been demonstrated in a great variety of cells (Glücksmann 1941, Hamazaki 1954, Lea 1955). In this study, a comparable change in cells of the transmissible venereal tumor of dogs followed x-ray irradiation. An almost complete disappearance and a subsequent reappearance of mitoses were demonstrated three to six hours and twelve hours, respectively, after the first irradiation at

dosage levels of 200, 300 or 400 r.

Since the majority of mitotic aberrations observed twelve to forty-eight hours after the first irradiation occurred during the prophase or metaphase rather than during the anaphase or telophase, it would appear that most of the tumor cells exposed to irradiation died during the stages of prophase or metaphase without completing the subsequent mitotic division. These findings correspond with the theory that when a cell is killed as a result of irradiation, death does not occur immediately but during or following the next division that the cell undergoes (Glücksmann 1941, Lea 1955).

An increase in the size of cells, or of their nuclei, following irradiation has been observed in a variety of tissues including human tumors (Glücksmann 1941, Wood 1949, Lea 1955). In the present investigation cellular and nuclear enlargement of the tumor was seen twelve to forty-eight hours after the first irradiation, and it became more pronounced after the second exposure. The average diameters of these cells and nuclei were one and a half times as large as those measured before irradiation. The degenerative changes observed in almost all cells following cellular and nuclear enlargement would suggest that the latter alterations represent an initial manifestation of cellular degeneration induced by irradiation. Furthermore, it would appear from these studies that when cellular and nuclear enlargement with nuclear disintegration have become pronounced (eg.: following four to six exposures to x-ray), cellular degeneration may occur directly rather than during a subsequent mitosis.

A peculiar histological change reminiscent of a perithelioma was observed early in the treatment of one case. The lesions consisted of agglomerations of intact tumor cells around small vessels. It seems possible that the nutrient supply of tumor cells lying remote from the capillaries was insufficient to sustain cellular function against radiation-induced damage whereas cells located adjacent to the capillary vessels survived.

The significance of stromal responses during radiation treatment of the tumor has been recognized by many investigators (Murphy 1923, Koller 1946, Windholz 1947, Jolles 1950). According to their views, tumor regression induced by irradiation is initiated by intra-cellular responses (or direct radiation effects) including suppression of mitosis, cellular and nuclear enlargement, chromosome fragementation and nuclear disintegration. Following these changes, regression is manifested by inter-cellular responses (or indirect radiation effects) represented by reactions closely resembling inflammation, with hypertrophy of connective tissue elements resulting in the formation of fibrous tissue. The connective tissue responses observed in the tumor of dogs after irradiation treatment were essentially the same as those described above. In these tissues fibroblastic proliferation was observed

initially, followed by infiltration of white blood cells during the period when intra-cellular disintegration was most conspicuous; thereafter, degenerated tumor cells disappeared and fibrosis increased.

The present investigation did not demonstrate significant differences in the cellular degeneration or connective tissue proliferation attributable to differences in the dose levels of x-ray irradiation. The complete regression of the tumor following exposure to a total dose of 1200 to 2400 r would indicate that the venereal tumor of the dog is highly sensitive to irradiation. In view of this, the suggestion by De Monbreun (1934) that the venereal tumor of the dog may be of lymphocytic origin is of interest.

The tumor cell degeneration and stromal response became conspicuous after the third or fourth exposure. Thus, the remaining two exposures may have been excessive and unnecessary. Other experiments are now in progress in order to determine whether complete regression of the tumor can be induced with less therapeutic exposures or with a smaller total dose.

The survival of the tumor mass located deep in the vaginal lumen of dog No. 5 (Fig. 4, Fig. 17) was probably due to inadequate exposure to x-ray. Recent experiences in this department indicate that contact x-ray therapy or surface therapy with radium or radioactive cobalt needles via the vaginal cavity may be effective in such a case.

The elevated total white-cell counts of these dogs, probably due to the inflammatory reaction on the surface of the tumor, decreased to normal following the regression of the tumor. The usual hematologic changes produced by ordinary therapeutic doses of irradiation have been described (Minot 1924, Lasser 1954, Lavedan 1954) as follows: a transient neutrophilia followed by leukopenia and lymphopenia, and a corresponding eosinopenia followed by eosinophilia. In the present studies an initial temporary neutrophilia was observed in four cases, and an initial slight eosinopenia was observed in five cases. No subsequent leukopenia or eosinophilia could be disclosed. A modest decrease in hemoglobin content and hematocrit occurred during treatment; thereafter, these values soon returned to or above pre-treatment levels. The cause of this anemia has not been clarified. However, from the hematological and clinical findings, it seems probable that the systemic effects of irradiation in the doses employed were relatively minor.

Summary

The response of the transmissible venereal tumor of dogs to x-ray irradiation has been studied. Tumors of eight female dogs, in the vaginal wall adjacent to the labia, disappeared completely following radiation therapy given in six exposures of 200, 300 or 400r over a period of three weeks (1200 to 2400r total dose). From

the results obtained it is concluded that the transmissible venereal tumor of the dog is sensitive to irradiation.

Histological studies revealed transient suppression of mitosis, cellular and nuclear enlargement and mitotic aberrations in the earlier stages of treatment. In the advanced stages of tumor regression, nuclear disintegration, infiltration with granulocytes, lymphoid cells, histiocytes and plasma cells followed by proliferation of fibrous tissue were observed.

Both clinical and hematological observations indicate that the irradiation therapy was well tolerated.

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Explanation of Figures (Plates XXII—XXV)

Fig. 1. Appearance of the tumor of dog No. 1 before irradiation treatment. The tumor developed over the vaginal wall near the vulva protruding between the labia.

Fig. 2. Degenerating tumor of dog No. 1 after four exposures of 200 r of X-ray.

Fig. 3. Complete regression of the tumor of dog No. 1 after six exposures of 200 r (1200 r total dose).

Fig. 4. The unaffected tumor mass adjacent to the cervix of dog No. 5. following the irradiation treatment. Note the regressed tumor near the vulva. t: unaffected tumor, u: uterus, r: regressed tumor, b: bladder.

Fig. 5. External appearance of the tumor of dog No. 7 before treatment. The tumor which developed all over the vagninal wall protruded from the vulva, elevating the perineum.

Fig. 6. Complete regression of the tumor of dog No. 7 after six exposures of 400 r (2400 r total dose).

Fig. 7-Fig. 9. Photomicrographs of the tumor of dog No. 7 before irradiation. Parenchymal cells with round or oval nuclei containing a distinct nucleolus are intermingled with a fine network of stromal fibers. Several mitotic figures are observed. Fig. 7 H-E, ×200, Fig. 8 Azan, ×200, Fig. 9 H-E, ×400.

Fig. 10. The tumor tissue of dog No. 7 biopsied forty-eight hours after the first irradiation. Abnormal mitotic figures including fragmented and sticky chromosomes are observed. H-E, × 400.

Fig. 11. Pronounced degeneration of the tumor cells of dog No. 7, four days after the second irradiation. Note cellular and nuclear enlargement and many pyknotic and karyolytic figures of the tumor cells. Scattered and fragmented chromosomes are observed in dividing cells. H-E, ×400.

Fig. 12. Nuclear disintegrations including vacuolization, karyolysis or pyknosis were noted in dog No. 7 three days after the fourth irradiation. Cell infiltration and fibroblastic proliferation became marked. H-E, ×400.

Fig. 13. Fat droplets in degenerated tumor cells of dog No. 7 three days after the fourth irradiation. Sudan III, ×400.

Fig. 14. Conspicuous fibroblastic proliferation associated with the advanced degeneration of tumor cells of dog No. 2, three days after the fourth irradiation. H-E, ×400.

Fig. 15. Prevailing fibrosis observed in dog No. 7 after the completion of treatment. H-E, ×200.

Fig. 16. Perivascular cuffs of tumor cells observed in dog No. 8 forty-eight hours after the first irradiation. H-E, ×100.

Fig. 17. An unaffected tumor tissue in the remained tumor mass located adjacent to the cervix of dog No. 5. H-E, ×200.

Fig. 18. Fibrous tissue near the vulva of dog No. 5. H-E, ×200.

Fig. 19. A chromosomal bridge observed in dog No. 7 immediately after the first irradiation. Heidenhain's iron hematoxylin, ×1500.

Fig. 20. Sticky chromosomes observed in dog No. 2 forty-eight hours after the first irradiation. H-E, ×1000.

Fig. 21. A tripple cleavage observed in dog No. 1, forty-eight hours after the first irradiation. H-E, ×1000.

Fig. 22. Chromosomal bridges observed in dog No. 7, four days after the second irradiation. H-E, ×1000.

Fig. 23. Scattered chromosomes in two mitotic cells observed in dog No. 7, four days after the second irradiation. H-E, ×1000.

Fig. 24. A ring-shaped histiocyte phagocyting cell debris observed in dog No. 2, two days after the third irradiation. H-E, ×1000.

要　　旨

犬の可移植性性器腫瘍におよぼすX線照射の効果

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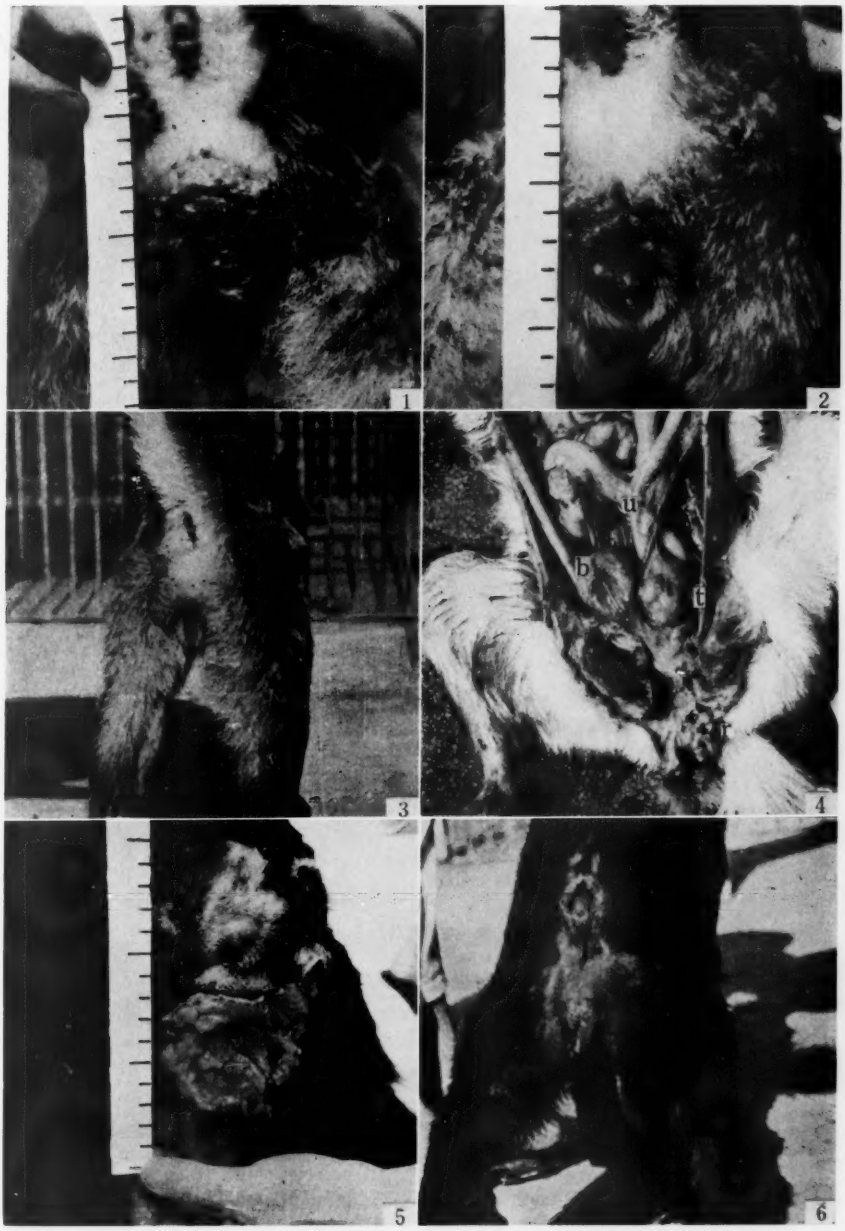
犬の可移植性性器腫瘍におよぼすX線照射の効果を，自然発生の本腫瘍を膣に有する8頭の犬について検索した。

陰門部附近に発生した腫瘍はすべて1回線量 200, 300, 400r の6回照射 (間隔 2～3 日) 総量 1200～2400 r の照射によって縮少治癒した。この結果本腫瘍が放射線に対する感受性の高い腫瘍であることが分った。

また組織学的検索の結果，治療初期に一時的な細胞分裂の抑制，種々の異常分裂像，細胞および核の大きさの増大が認められた。それ以後の時期では腫瘍細胞の著しい変性，遊走細胞の侵潤が認められ，線維芽球の増生によって腫瘍基底部に瘢痕組織が形成された。

臨床所見および血液所見から考えるとX線照射の犬の全身状態におよぼす影響は少なかった。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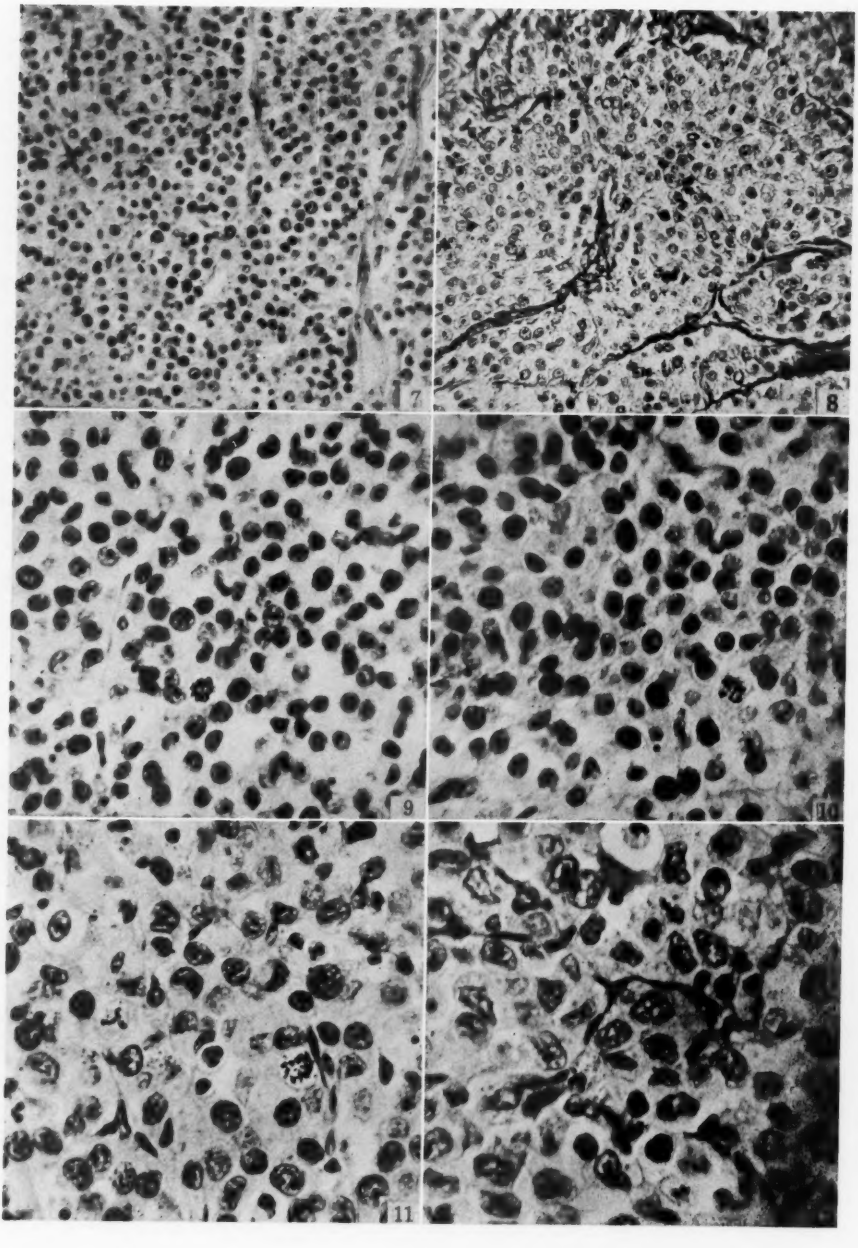
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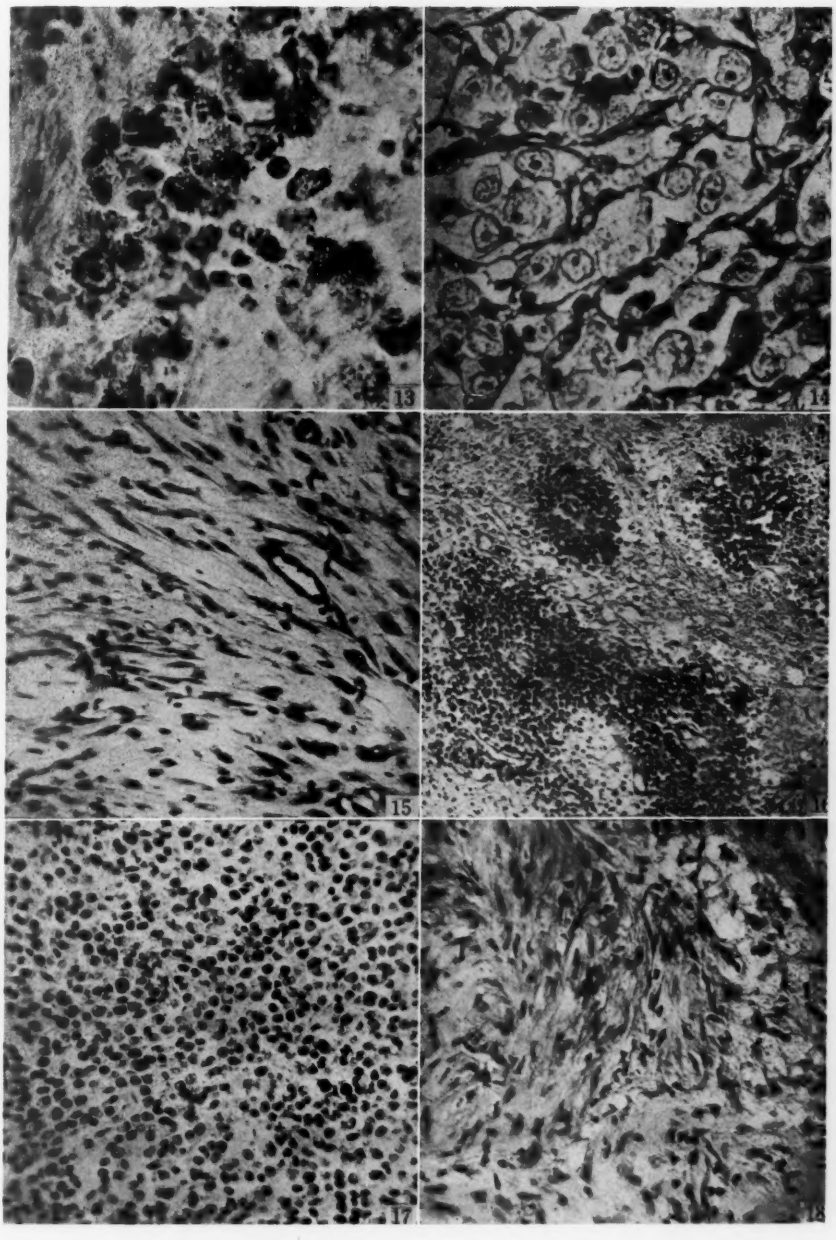
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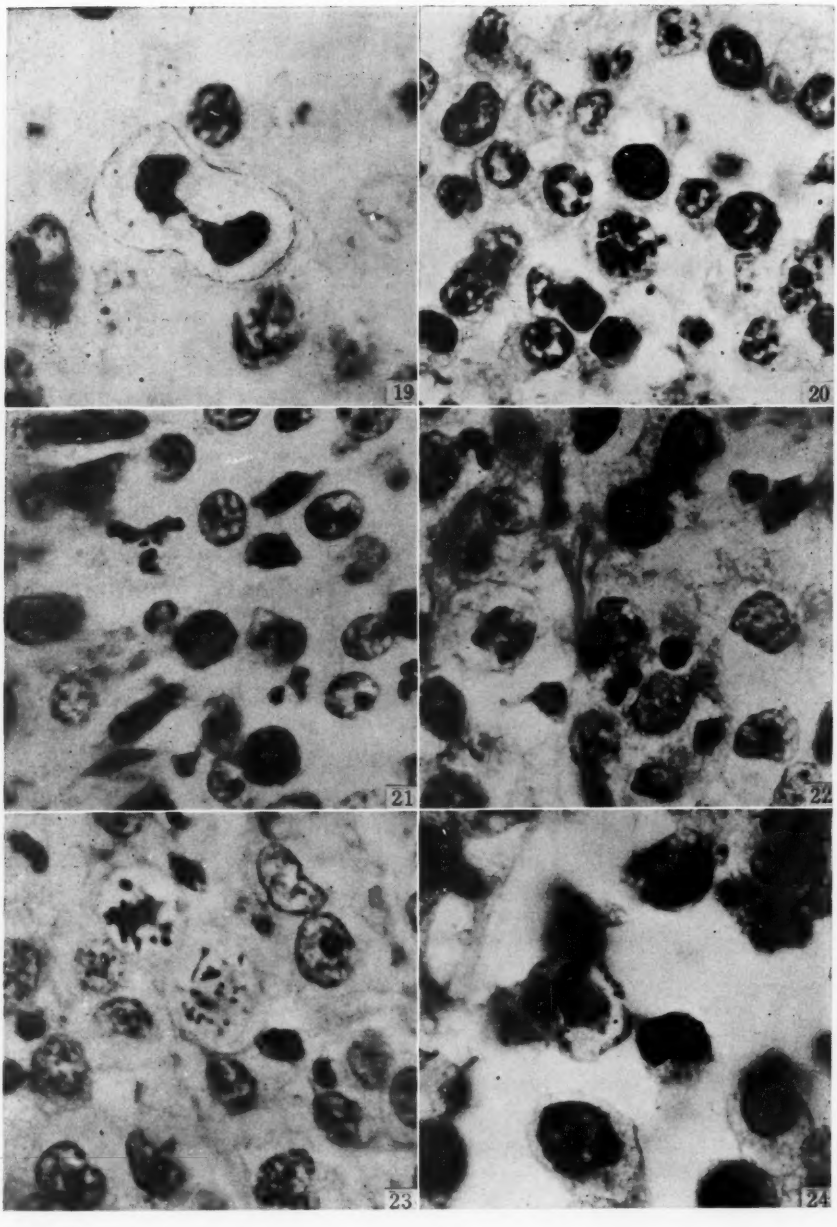
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[GANN, 49, 319~330; December, 1958]

CHANGES IN GLYCOGEN, RNA AND DNA CONTENTS IN LIVERS OF TUMOR-BEARING RATS

(Plate XXVI)

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The pathological and biochemical changes of tumor-bearing animals in comparison with those of normal ones have been a subject of repeated studies in the field of oncology. The inhibition of catalase in the liver (Appleman *et al.* 1950, Dounce & Shanewise 1950, Greenfield & Meister 1951, and others) and the increment of P^{32} incorporation into tissue deoxyribonucleic acid (DNA) (Payne *et al.* 1952, Kelly *et al.* 1951 and others) have been claimed as a sequence of tumor transplantation. In the course of cytochemical studies on the mitochondrial morphology in relation to chemical constituents in rat liver cells, one of the authors (Hori 1958) has found that the almost complete depletion of glycogen occurs accompanying a considerable increase of cellular basophilia in the liver of a tumor-bearing animal without remarkable alteration in morphology of the mitochondria. In the precent study, the authors have undertaken to inquire in detail into what changes do occur in contents of glycogen, RNA and DNA in the liver of rats bearing tumor transplants. Microspectrophotometry of the DNA content was performed at the Kwansei Gakuin University under the direction of Professor Yoshio Ojima to whom the authors' thanks are due.

Material and Method

The MTK-sarcoma III was inoculated intraperitoneally into Wistar rats weighing 70 to 120 gm. Rats bearing tumors were sacrificed 3, 6 and 8 or 9 days after the tumor inoculation; they are conveniently referred to as Group-A, -B and -C rats, respectively, in the following descriptions. The mean survival time of the hosts was 8.7 days. The livers from normal and tumor-bearing rats were fixed with formal-calcium and Gendre's fluid, and subjected to a series of cytochemical tests as follows:

Periodic acid Schiff reaction (PAS) according to the method of McManus (1948) was applied to the material which was fixed with subzero Gendre's fluid for the

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demonstration of glycogen. The Feulgen reaction according to Stowell's method for DNA, the toluidine blue staining method for RNA followed with or without the digestion test by ribonuclease (Worthington Biochem. Sales Co., Freefold, New Jersey, U.S.A.*), the bromphenol-blue method for basic protein according to Mazia (1953), and the light green method for protein, with or without hydrolyzation by HCl (Kaufmann *et al.* 1951), were applied to sectioned materials fixed with formal-calcium. Microspectrophotometric measurement of DNA in liver nuclei was performed according to Swift's method (1950). Regaud's staining was also applied to formal-calcium fixed tissues which were chromated with 3% potassium bichromate for 6 days at room temperature. Fixation with formal-calcium yielded good results as well as Regaud's original fixative consisting of formal and bichromate.

Observations

Periodic acid Schiff reaction and histological remarks: The cytoplasm of liver cells of well-nourished healthy rats was generally well-supplied with glycogen, the distribution of which was almost uniform throughout hepatic lobules (Fig. 5).

The livers from Group-A rats (sacrificed 3 days after the tumor transfer) contained PAS positive substances in much less amount than the controls. Through the digestion of sectioned material with saliva extract, the PAS positive substance was shown to be glycogen. Depletion of glycogen was observed in all of the hepatic lobules (Fig. 6), being especially remarkable in their periphery. Mitoses of hepatic sinusoids were also remarkable. The leucocytes were abundant among the anastomosing hepatic strands than in the controls.

In the material (Group-B) six days after the tumor transplantation, the liver was found mostly covered with a layer consisting of necrosed and viable tumor cells having the cytoplasm strongly positive to the PAS reaction (Fig. 12). Division of hepatic cells took place more frequently than in the former group. PAS reaction revealed further advanced depletion of glycogen in the livers (Fig. 7). Glycogen granules were mostly invisible in the cells at periphery of the lobules, while the centrolobular cells contained the granules in the form of globules or minute particles. The mitotic apparatus of hepatic cells was completely negative to the PAS reaction. The leucocytes in the hepatic sinusoid were more numerous in Group-B rats. The cytoplasm of the leucocytes showed slightly diffused red coloration when the PAS reaction was applied.

All of the livers from Group-C rats are characterized by cells which contained a small amount of glycogen. Certain liver cells contained only a single minute PAS positive granule in each (Fig. 8). Leucocytes in the sinusoid further increased in number and their cytoplasm was strongly positive to the PAS reaction. A con-

* Kindly supplid by Dr. C. Leuchtenberger for the use of this study. The anothors' cordial thanks are due to her cooperation.

siderably strong tissue reaction was observed throughout the whole liver with a remarkable dilatation of sinusoids. Consequently the original architecture of the hepatic lobules was distorted. Mitoses of hepatic cells were frequent, though they seemed to be a little less in number than those in the livers of Group-B rats. Another remarkable item of change occurring in the liver of Group-C rats was a striking increase in stainability of hepatic cell nuclei with toluidine blue.

The toluidine blue staining: Examinations of the material stained with 0.1% toluidine blue solution have revealed that both the cytoplasm and nucleolus of the hepatic cell show blue coloration while their nuclear chromatin is metachromatically blue-violet (Fig. 1). After the treatment with 0.02% ribonuclease solution the stainability of the cytoplasm and nucleolus was completely lost, whereas the coloration of the chromatin changed from blue-violet to violet. This seems to imply that blue coloration obtained after toluidine blue staining is owing to the existence of RNA in the cellular constituents.

In comparison with the control rats the tumor-bearing rats show an increase in amount of RNA in the cytoplasm of hepatic cells. Such an increase was evident in the livers of Group-A rats and much more remarkable in those of Group-B and -C rats (Figs. 2-4). The cytoplasm contained a number of mitochondria-like bodies which were spherical and rod-like in shape. The peripheral zone of the bodies was stained blue and rather translucent in the inner part. Some of them occurred in close contact with the nuclear membrane while others aggreagated into several masses scattered in the cytoplasm. The stainability of the nuclear membrane and chromatin, especially nucleolus-associated chromatin, was also strong in the livers of the tumor-bearing rats. The nucleoli increased both in number and volume. Generally they were irregularly shaped and often in contact with the nuclear membrane. The nucleolar or chromatic substance adhered to the inner surface of the nuclear membrane, so that the membrane displayed an abnormal thickening. The cytoplasm adjacent to the nuclear membrane stained blue in most cases more remarkably than the other cytoplasmic areas. Sometimes, the thick nuclear membrane extruded somewhat into the cytoplasm, thus bringing about a wavy and irregular surface of nuclei. Such a feature of hepatic cells was rather common in the tumor-bearing rats (Figs. 9 & 10). Based on the above findings the following speculation may be allowable that the presence of a tumor in the rats induces the metabolic activity of their hepatic cells to produce a large amount of RNA, and further that the increase of RNA amount in those cells may be closely related to the accelerated elaboration of the nucleus.

The hepatic nuclei of Group-C rats are generally characterized by a very strong affinity to the basic dye (Fig. 4). The majority of nuclei of this Group were rather small in size and densely stained with toluidine blue. It is therefore rather difficult

to study in detail the morphology of the nucleoli and chromatin in those cells. There was no evidence of the extrusion of nucleoli in those nuclei.

Regaud's iron-hematoxylin staining: It was aimed by the employment of this method to demonstrate the extrusion of the nucleolus in hepatic cells of tumor-bearing rats, since the evidence was expected to be found by the examination of the toluidine blue preparations. The examination failed however to demonstrate the extrusion of nucleoli. Instances of the thickening of the nuclear membrane, probably caused by the association with chromatic or nucleolar substance, and the existence of nucleoli of polymorphic nature were found to occur in the preparations here studied, in a similar way as in the toluidine blue preparations.

Staining with light green and bromphenol blue: These two methods for examination of test of basic protein did not yield any appreciable results: little difference was observed in stainability between the control and experimental materials.

Feulgen nucleal reaction: Upon examination of Feulgen stained preparations particular attention was concentrated on the possibility of extrusion of the nuclear DNA into the cytoplasm, on the assumption that if the extrusion of nucleolar material would occur concomittantly with the liberation of chromatin, especially nucleolus-associated chromatin, from the nucleus, the Feulgen positive substance should be found in the cytoplasm. Contrary to the expectation, no Feulgen positive material was detected in the cytoplasm either in control or in rats bearing tumor transplants.

Microspectrophotometric measurements of the DNA contents in liver cell nuclei: The DNA contents in liver cell nuclei of tumor-bearing rats were measured in formal-fixed and Feulgen-stained preparations by means of Swift's type apparatus. The results obtained are collected in Text-figure 1: four histograms at the left are presented by way of comparison of the data between the liver of a newborn rat and the livers of three tumor-bearing rats[1] at the 9 th day of tumor inoculation. The histograms at the right show the data from the liver of a normal rat in comparison with those from the livers of three rats which were killed 3, 6 and 8 days after the tumor transplantation[2]. Measurements of the DNA contents in these two groups were carried out at different times and by different present authors. Accordingly, arbitrary units of the DNA contents used are slightly different by measurements.

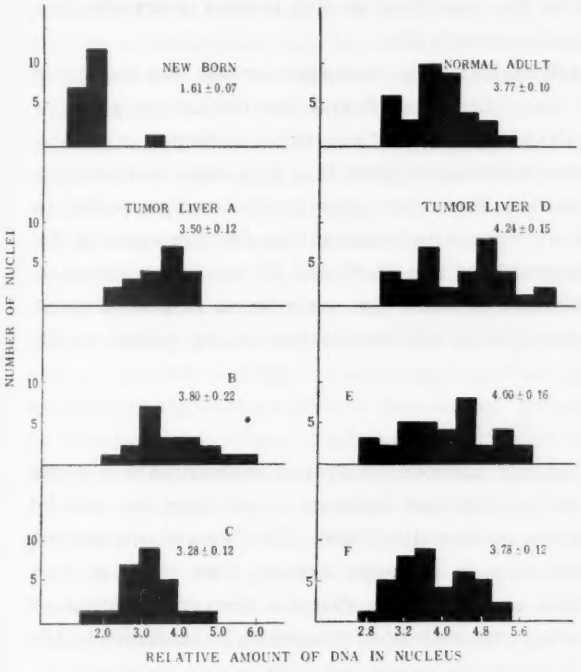
Comparison of DNA amount between the newborn and tumor livers A, B and C: It has been widely accepted that the DNA amount of most liver cells of adult rats is twice the amount of those of the newborn rat. If the average amount of DNA

1) They are conveniently referred to as newborn liver and tumor livers A, B and C in the histograms.

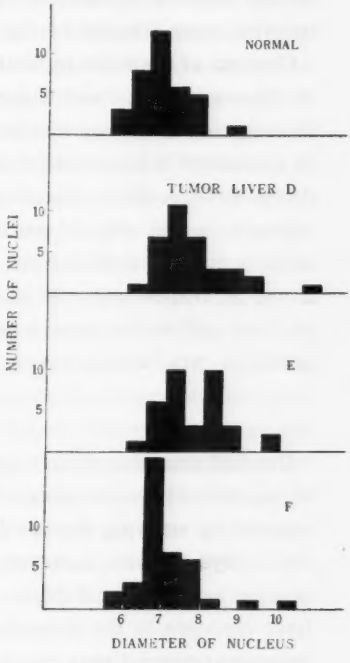
2) They are indicated as tumor-livers D, E and F in the histograms.

(1.6) in the newborn liver here estimated is assumed to be a diploid value (2x), its duplication (4x) is 3.2. Tumor liver A and B show a slightly higher average value than the expected 4x value, while tumor liver C shows the expected 4x value.

Comparison of DNA amount between the normal adult and tumor livers D, E and F: A normal liver and tumor liver F, which was obtained from a rat killed



Text-Figure I. Four histogram at left hand indicate relative amount of DNA in the liver cells of a newborn rat, and of three tumor-bearing rats at the 9th day of tumor inoculation, respectively. The histograms at right hand show relative amount of DNA in the liver cells of a normal adult rat, and of three tumor-bearing rats which were killed 3, 6 and 8 days after the tumor transplantation, respectively. For detail see text.



Text-figure II. The histograms show nuclear diameters of the liver cells of a normal adult rat, and of three tumor-bearing rats which were killed 3, 6 and 8 days after the tumor transplantation. For detail see text.

8 days after the tumor transfer, showed a similar pattern of frequency distribution of the DNA content; the average value of DNA content is 3.77 ± 0.10 and 3.78 ± 0.12, for the normal and tumor liver F respectively. On the contrary, the DNA contents of tumor livers D and E which were obtained from rats killed 3 and 6 days after tumor inoculation, were slightly higher than those of the normal and tumor liver F, and the scatter ranges of DNA contents in the former two samples were also wider than in the latter ones. The standard deviation was 0.91 and 0.97 in the

former, and 0.62 and 0.73 in the latter, respectively.

On the basis of the above findings it is highly probable that the tumor exerts influence upon the host liver to raise the DNA content in its liver cells during the period of the most active growth of the tumor which ranges from about 3 to 6 days after tumor transfer, with or without a return of the DNA content to the normal value which occurs during the time from the 8th to the 9th day after the transfer, near the end of the tumor-animal's life.

Changes of diameter in liver cell nuclei: The diameter of nuclei was measured in the normal liver and tumor livers D, E and F and the results are given in Text-figure II; the mean in length of long and short axes of the nucleus is expressed as a diameter in an arbitrary unit. Referring to these data it is clear that changes in the DNA content occurring during the tumor growth run closely parallel to changes in the size of nuclei of liver cells: namely, the average value of the nuclear diamter is 68 for the normal liver and 76, 77 and 70 for tumor livers D, E and F, respectively. It is therefore probable that an increase in DNA content of liver cell nuclei occurs concomittantly with an increase in the volume of cell nuclei in rats bearing tumor.

Discussion

Gradual decrease of liver glycogen in tumor-bearing rats was observed in a series of experiments in the present study. Glycogen depletion in rat livers can also be induced by starving the rat for several days (Hori 1958). The livers of rats starved for 6 days showed, however, much more glycogen contents than those of rats bearing a tumor for 6 days. This seems to imply that the observed depletion of liver glycogen in the tumor-bearing rats may be attributed to, or be caused by the transplantation of that tumor.

There are a number of studies which indicate that the carbohydrate-containing proteins in serum increase beyond their normal levels both in patients with cancer and in animals bearing transplanted tumors (cf. Baldwin and Harries 1958). Probably, the liver glycogen may play a role in relation to the increase of serum carbohydrate-containing proteins, and further the marked depletion of liver glycogen as observed in the present study may be responsible for the increase of serum carbohydrate-containing proteins as above dealt with.

Blood sugar level and liver glycogen are under the control of various kinds of hormones, such as adrenalin, insulin, anterior pituitary and thyroid hormones, so that the disturbance of hormonic balance following the injury of endocrine glands which can be induced by various factors must be taken into consideration, so far as the fate of liver glycogen is concerned. Dalton and Peter (1944) studied the effects of tumor on the endocrine glands, and reported that marked depletion of

lipids occurred in the adrenal cortex of tumor-bearing mice. Umeda *et al.* (1957) observed involution of the thymus which was accompanied by a decrease in DNA and RNA contents. An increase of glycolysis has also been reported in the liver of leukemic mice by Hall (1944) and Burk *et al.* (1942).

Bennett (1956) proposed an interesting hypothesis pertaining to the transmission of nucleoprotein from nucleus to cytoplasm, indicating that some of the RNA newly formed in the nucleus may become attached to the inner nuclear membrane and be carried out onto the ergastolasmic portions of the endoplasmic reticulum by the "membrane flow." In the electron micrography study of salivary-gland cells of third instar larvae of ***Drosophila melanogaster***, Gay (1956) has observed nuclear membrane out-pocketings and regarded them as a mechanism for transport of materials of chromosomal origin into the cytoplasm. Moses (1956) also observed out-pocketings of the nuclear envelope in a crayfish spermatid. Using a hemipteran insect as material, Anderson and Beams (1956) obtained electron micrographs which show that actual transport of nuclear substance takes place through pores of the nuclear membrane to the cytoplasm. As referred to above, evidence favorable to the idea of transport of nuclear material into cytoplasm is now available specially in the field of electron microscopy. In the present study, observations of toluidine blue stained preparations furnished evidence that basophilia of the hepatic cells increases to a great extent in tumor-bearing rats. In addition to the increase of cytoplasmic basophily, the extrusion of nucleolar material and its hypertrophy observed in the hepatic cells of tumor-bearing rats seem to be evidence supporting the view that the transport of nuclear material, especially RNA, into the cytoplasm occurs in the livers, though no conclusive statement can be made without further supplementary experiments.

It has been shown that the tumor transplanted into animals exerts influence upon the turnover of tissue deoxyribonucleic acid. Payne *et al.* (1952) reported an increase of P^{32} incorporation into the liver DNA in rats bearing tumor transplants by way of comparison with the control animal. Kelly *et al.* (1950) and Kelly and Jones (1950) also obtained similar results. Effects of tumor transplants on nucleic acid content of host mouse tissues were investigated by Reddy and Ceredo (1951). They found that both DNA and PNA increased in the livers of mice bearing Crocker sarcoma 180 transplants, and regarded such a change as the above to be a result of increased cell proliferation.

The present study has yielded results indicating a slight, but significant increase of net DNA contents in liver cells of animals with tumor transplants. Though it seems difficult to expect accurate results in measurement of DNA contents by means of microspectrophotometry, the above evidence receives support from the finding that an increase in nuclear volume of liver cells of tumor-animals occurs

concomittantly with an increase in DNA contents of those cells.

Yeakel (1948) and Yeakel and Tobias (1951) reported that liver weight increased in rats bearing induced or transplanted tumors. Annau *et al.* (1951) also found an increased weight and mitotic activity in the liver of tumor-bearing rats and mice, and concluded that the increase in weight might be partly a consequence of mitotic activity induced by the presence of the tumor.

The increased mitotic activity in livers of tumor animals may be associated with the increased DNA content in liver cells observed in the present study, though there is no direct evidence to support that interpretation.

Since Vendrely and Vendrely (1948) found that the DNA content of isolated liver nuclei is approximately twice that of spermatozoa, a number of reports have been published pertaining to the DNA contents of various kinds of cells. Many authors agree that the DNA content of a nucleus is strictly correlated with its chromosome number (Alfert & Swift 1953, Davidson & Leslie 1950, Mirsky & Ris 1951, Thomson and Frazer 1954, Cole & Leuchtenberger 1956 and many others, see also the review by C. & R. Vendrely 1956), while some authors hold a view contrary to the above (Pasteels & Lison 1950, Lison & Pasteels 1951, Fautrez & Fautrez-Firlefyn 1953). According to them, the DNA content of the nucleus can vary with the physiological activity of the cell. Recently, several reports have become available regarding the views of Belgian workers. Lowe and Rand (1956) investigated the effect of cortisone on the DNA content of rat liver cells; they reported that the DNA content of liver nuclei fell during cortisone administration, the fall was progressive with continuity of hormone administration (by 20%), and the return to normal occurred 3 days after discontinuance of cortisone administration. LaCour *et al.* (1956) observed a considerable drop in the DNA content of the nuclei after exposure of plant root meristematic cells to low and high temperature. Leuchtenberger *et al.* (1956) observed in dwarf bulls a considerable variation of the DNA content per nucleus during spermatogenesis which is not followed by the shift of the chromosome number.

Bendich (1952) and Bendich *et al.* (1953) discovered the existence of different DNA fractions, DNA_1 and DNA_2, which show significant metabolic differences. According to them, the ratios of DNA_1 to DNA_2 may be related to the mitotic activity of the tissue from which the DNAs were derived. Morin *et al.* (1956) also reported the metabolic heterogeneity of DNA. They examined the incorporation of P^{32} into the DNA of the various fractions which were isolated by the method of differential centrifugation from mouse liver nuclei, and found evidence that the fractions rich in nucleolus-associated chromatin showed the highest specific activity among the fractions isolated. There are still additional reports which oppose the review as to the strict constancy and metabolic stability of DNA in the nucleus, or as to the

close correlation between the chromosome number and the DNA content per nucleus.

In reference to the reported evidence as above, it seems that the deviation of DNA content from the values which are expected from the chromosomal ploidy basis can not be discussed on the basis of mere change in the DNA content of the nucleus occurring during the mitotic cycle.

Fautrez (1956) and Fautrez *et al.* (1956) reexamined the correlation between the volume and DNA content of individual nuclei in different tissues, of the rat, and presented evidence that the average nuclear volumes for their three given classes (2C, 4C and 8C) follow exactly the same progression as the average DNA content. They showed further that within one of those classes the relation between the DNA content and volume is not very evident for the different individual nuclei, though there exists a relation at least to a certain extent.

If an increase of DNA contents with an increase of nuclear volume as observed in the present study should be related to change in DNA content of the nucleus during the mitotic cycle, an increase in nuclear volume should in turn occur during the mitotic cycle. In other words, if a close correlation should exist between the nuclear volume and DNA content, the volume of the nucleus just undergoing division should be twice that of the nucleus at the end of division. There is no direct evidence for the above supposition, and therefore, the present findings showing that the DNA contents and nuclear volume increased in liver cells of the tumor-bearing animals remain without clean explanation at present.

Wolstenholme and Gardner (1950) investigated histologically the livers of mice with transplanted testicular tumors and described the sinusoidal dilatation as usual while the destruction of the normal lobular architecture of the liver was induced by the dilatation. This is parallel to the present findings.

In conclusion, the marked depletion of glycogen, the gradual increase of RNA contents, the significant increase of DNA contents with a return to normal contents in the latter part of the tumor-animal's life, the increase of mitotic activity and the sinusoidal dilatation are a series of findings which have been derived from the observations of the liver cells of rats bearing tumor transplants. However, the authors hesitate to conclude simply that the existence of the tumor directly induces these changes, since some changes of similar character not uncommonly occur in rats without tumor when they are exposed to certain physiological and pathological conditions.

Summary

In the rats which received the transplantation of the ascites tumor, MTK-sarcoma III, the changes in contents of glycogen, RNA and DNA in the liver cells were investigated by means of a series of cytochemical and microspectrophotometric methods.

Gradual glycogen depletion was consistently observed from experiment to experiment in the liver cells of rats bearing tumor transplants. Every rat which died of tumor contained a very little amount of glycogen in their liver cells. The rate of the glycogen depletion of tumor-bearing animals exceedingly surpassed that of starved rats. It has remained unsolved in the present study whether the observed depletion of liver glycogen occurred in rats as a direct response to the enormous requirement of energy by tumor cells, or whether tumor transplants attacked hormonal glands of the host which control the glycogen storage in the liver. Whatever the causes may be, it is very likely that the glycogen depletion observed in the tumor-bearing rats may be attributable to the tumor transplants in hosts.

Contrary to the glycogen depletion, RNA (basophilia digestable by RNase) increased gradually with the prolonged existence of the tumor transplants. The increase in amount of RNA was observed both in the cytoplasm and in the nucleus. The thickening of the nuclear membrane and the hyperactivity of the nucleolus observed in the tumor animals seem to indicate the active participation of the nucleus in association with the accelerated production of RNA in the liver cells.

An increase of net DNA content (Feulgen-Schiff complex) took place in connection with an increase of the nuclear volume in liver cells of tumor-bearing animals during the period of the most active proliferation of the tumor. Its significance was discussed in relation to the DNA content of the nucleus.

Increased mitotic activity and dilatation of the liver sinusoids were also observed in livers of the rats bearing tumor transplants.

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Explantation of Plate XX

Figs. 1–4. *Fixative*: formal-calcium. *Stain*: toluidine blue. *Magnif.*, 400×.

Fig. 1. Control rat liver.

Figs. 2–4 livers of tumor-bearing rats sacrificed on 3, 6 and 8 days after transplantation, respectively.

Figs. 5–8. *Fixative*: subzero Gendre's fluid. *Stain*: periodic acid Schiff. *Magnif.* 100×, except Fig. 8 which is 400×.

Fig. 5. Control rat liver. Glycogen abundant, showing a uniform distribution throughout the hepatic lobules.

Figs. 6–8. Livers of tumor-bearing rats sacrificed on 3, 6 and 8 days after transplantation, respectively.

Note a gradual decrease of PAS positive substance in liver cells.

Figs. 9–11. *Fixative*: formal-calcium. *Stain*: toluidine blue. *Magnif.*, 1000×. Liver cells from the tumor-bearing rats.

Figs. 9 and 10. Showing the extrusion of nucleoli into the cytoplasm.

Fig. 11. Nuclei stained densely with toluidine blue.

Fig. 12. *Fixative*: Gendre's fluid. *Stain*: PAS. *Magnif.*, 400×. Showing tumor cells in contact with the surface of the liver, 6 days after the tumor transplantation. Note the positive reaction of the cytoplasm.

Fig. 13. *Fixative*: formal-calcium followed by the chromation with potassium bichromate for 6 days. *Stain*: Regaud's iron-hematoxylin. *Magnif.*, 1000×. Liver cells of tumor-bearing rat 6 days after transplantation. Mitochondria show no marked morphological change by the presence of the tumor.

Addendum. After this article was completed and sent to press, Leuchtenberger, Leuchtenberger and Uyeki (1958) published an article: Cytological and cytochemical changes in livers of white mice following intraperitoneal injections of DNA preparations from breast cancers of agouti C_3H mice (Proc. Nat. Acad. Sci. 44: 700–705), in which they present approximately similar results to those obtained in this study, so far as the DNA contents are concerned.

要　　旨

MTK-肉腫 III を移植せるシロネズミ肝のグリコーゲン, RNA, および DNA の消長について

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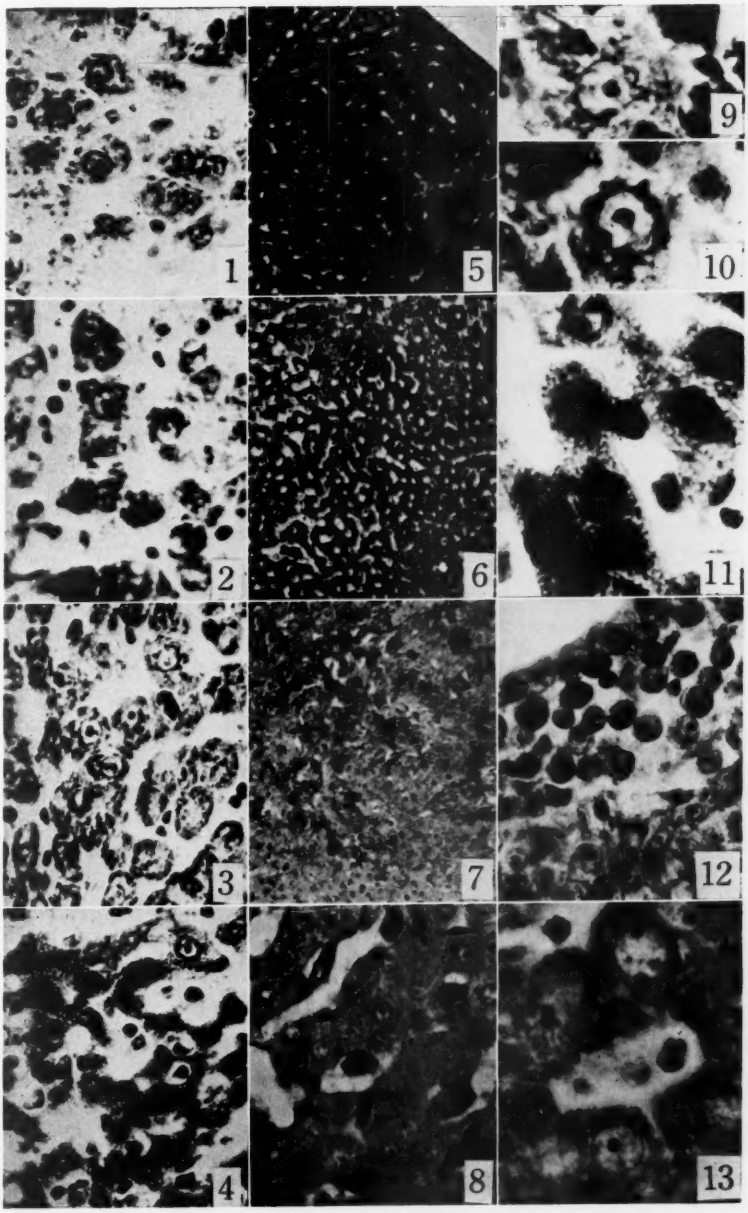
(北海道大学理学部動物学教室)

Wistar 系シロネズミ (70～120g) の腹腔内に MTK 腹水肉腫 III を移植し，移植後 3, 6, 8 または 9 日後に宿主を殺して，肝におけるグリコーゲン (McManus PAS 反応)，RNA (Toluidine blue 染色) および DNA (Feulgen-Schiff 複合体の顕微測光法による測定) の消長を調査した。

グリコーゲンは正常なネズミの肝においては常に豊富に含有されているが，肉腫を移植されたものにおいては，次第に肝小葉周辺部より減少し，宿主が死亡する 8～9 日後にはほとんど肝全体に亘って消失してしまう。6 日間絶食させた，肉腫を植えないネズミにおいてもこれほど極度なグリコーゲンの消失は見られなかった。グリコーゲンの消失とは逆に，RNA は肉腫移植後次第に増加する。RNA の増加と関係があると思われるが，仁の肥大・多形化・数の増加および核膜の肥厚が肝細胞において一般的に観察された。一方，DNA も担癌動物において，やや増加する傾向が見られた。また肝細胞核の体積の増加も DNA 量の増加と平行して見られた。この外，担癌動物肝においては，有糸分裂の増加および sinusoid の膨脹が観察された。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[GANN, 49, 331~340; December, 1958]

CYTOLOGICAL STUDIES OF TUMORS, XXIV. AN AZO-DYE INDUCED ASCITES HEPATOMA OF THE RAT, WITH SPECIAL REMARKS ON TRANSITIONAL CHANGES OF NEOPLASTIC CHARACTERS IN THE COURSE OF SERIAL TRANSFERS

(Plates XXVII and XXVIII)

MOTOMICHI SASAKI

(Zoological Institute, Hokkaido University)

It has generally been known that transplantable tumors of rats and mice maintain in general a very pronounced constancy keeping their cytological and neoplastic characteristics during successive transfers. Recent investigations have revealed the fact that under certain conditions, however, the stability is not always permanent and some transitions occur in the stemline chromosomes of the neoplastic population with changes in the properties of the tumor (Levan 1956 a, b, Makino 1956, 1957 a, b, c, Tonomura and Sasaki 1957, Makino and Sasaki 1958). Since the tumor pattern is to be controlled by the tumor genotype, it is very likely that the development of a new tumor type with changes in the neoplastic property may be closely associated with chromosome changes in stem-cells.

In connection with the above considerations, the present author has studied the rise and further development of an ascites hepatoma induced by azo-dye application in a rat. The present paper gives some information about the shifts in the chromosome pattern of such an ascites hepatoma and some other transitional changes occurring in neoplastic characters during serial transfers.

The author wishes to express his sincere thanks to Professor Sajiro Makino for his direction and improvement of the manuscript for publication. Further cordial thanks are offered to Mr. T. Tanaka and Mrs. K. Kanô for their valuable advice and criticism. Thanks are extended to Dr. H. Kobayashi, Pathology Department, Medical School, for his kind direction in some pathological examinations of the material.

Contribution No. 422 from the Zoological Institute, Faculty of Science, Hokkaido University, Sapporo, Japan.

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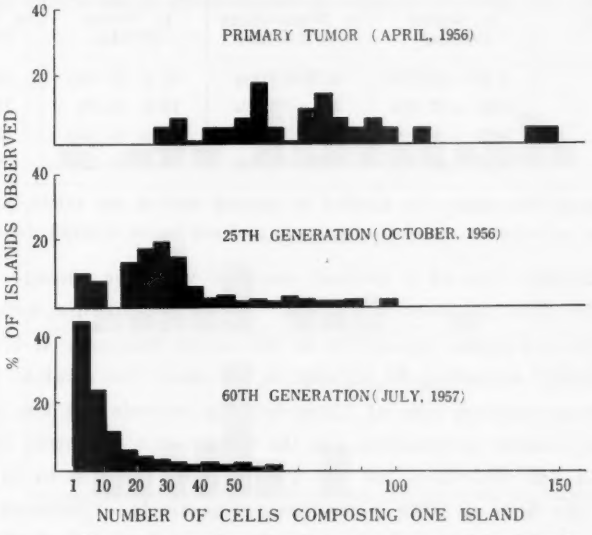
Material and Method

The hepatoma (H-17) to be described here is one of the transplantable ascites tumor lines in the rat. This tumor was originally produced in a highly purebred rat (Wistar-King A strain, ♀, F 153) by feeding p-dimethylaminoazobenzene for a period of 237 days. Primarily there was a development of anaplastic hepatoma in the liver (Fig. 10). With the formation of a solid tumor in the liver, the ascitic fluid containing a number of so-called hepatoma islands was produced in the peritoneal cavity (Fig. 11). The ascites was considerably hemorrhagic in nature. About 0.3 cc. of the ascitic suspension of tumor islands was injected in the peritoneal cavity of a new rat weighing 50 g (Wistar-hyphen King A strain, ♀). After injection the propagation of tumor cells occurred in the ascites of the rat. The serial transfers have beeen continued since its start in April 1956 for over 90 generations (March 1958). Two strains of purebred rats, Wistar-King A (WKA/Ma) and Wistar (W/Ma), were used for the serial transmissions of this tumor. Microscopical observations were made with temporary preparations of the ascites tumor stained with acetic dahlia. For observation of the chromosomes water-pretreatment squash method was adopted advantageously (Makino 1957 b). Sections of solid tumors were also prepared after fixation in 10 per cent formalin and stained according to the hematoxylin eosin method.

Observations

1. ***Tumor cells forming hepatoma islands***: The hepatoma islands in the primary tumor ascites were comparatively large in size and the cells forming each island were considerably adhesive with each other. The cells forming the surface layer of the island were flat and elongated in shape (Fig. 11). Mitotic cells were found in many islands, but the chromosomes were entirely obscure in appearance due to striking stickiness. The number of cells composing one island was roughly counted under microscope on the basis of 29 islands, but exact counting was very difficult, since the cell bodies were very fragile and the boundary of each island was not clear-cut. As shown in Text-figure 1, over 50 per cent of the islands observed were composed of 50 to 80 cells, while some were islands of over 100 cells. There were no islands containing cells less than 20 in number. With the increase of transfer generations the hepatoma islands showed a gradual change in feature; the size of the islands became smaller and smaller and the component cell elements showed a tendency to reduce their adhesiveness. In the samples derived from the 25 th generation (October 1956), about 67 per cent of island (148 out of 219 islands observed) contained 15 to 40 cells. There occurred a fair number of islands which consisted of less than 15 cells, though the primary tumor showed no such a small island at all as may be seen in Text-figure 1. Figure 12 shows

the general feature of the tumor ascites on the 5th day of the 25th transfer generation. There are loosely bounded cells with a reduced stickiness in the islands. The surface cells had decreased in flat appearance (Fig. 12). In the samples taken on the 5th day of transfer in the 60th generation (July 1956), islands composed of 1 to 15 cells showed over 80 per cent and there were no islands having more than 65 cells so far as 252 islands were observed (Text-fig. 1, Fig. 13). The small islands consisting of a few cells have remained in situ at the present time (90 generation, March 1958). It is thus evident that the size of hepatoma islands has undergone gradual transitions becoming smaller and smaller with the passage of transfer generations.



Text-figure 1. Change in number of cells composing one island in the ascites hopatoma (H-17) during successive transfer generations.

2. ***Thransplantability and tumor-animals' life span***: As described above, the ascites hepatomas (H-17) here under consideration were originally established in a highly purebred rat (WKA/Ma, F 153). In the early transfer generations, however, this tumor did not show lethal transplantabillty to rats of the mother strain, Wistar-King A. In the first 9 generations, tumorcel ls of the ascites hepatoma proliferated very slowly in the peritoneal cavity of the animals, showing a poor increase of ascites without attaining a state of pure culture. The proliferation of tumor cells stopped within 6 to 10 days after inoculation, and thereafter spontaneous regression of tumor cells followed. At autopsy no visible tumorous invation was found in the viscera. The tumor, however, could be transferred from rat to rat by repeating serial intraperitoneal transplantations. After 10 to 20

serial transfers from the start, the growth rate of the tumor cells increased gradually and there were several animals which died of tumor with its invasion into visceral tissues, showing a moderate increase of ascitic fluid in a state of nearly pure culture. For the lst to 20th transfer generations, the lethal transplantability of the tumor-bearing animals was only 23.1 per cent for the WKA/Ma rats and 26.7 per cent for the W/Ma rats. The mean life span of the tumor-animals was 19.0 days for the WKA/Ma rats and 37.5 days for the W/Ma rats (Table 1). Thereafter,

Table 1. Changes of survival time and mortality of rats bearing the ascites hepatoma (H-17) in the course of 80 transplant generations.

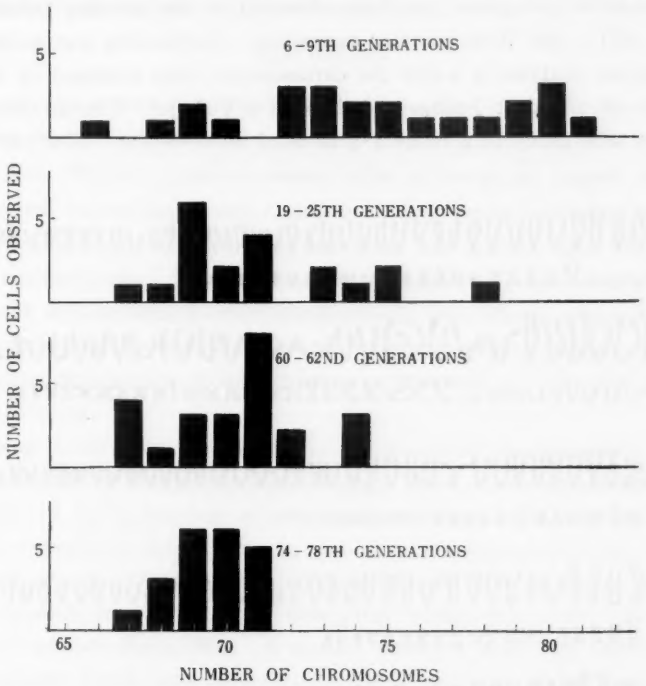
Transplant generation	Transplantability		Mean life span, in days (Range)	
	in Wistar (W/Ma)	in Wistar-King (WKA/Ma)	in Wistar (W/Ma)	in Wistar-King (WKA/Ma)
1-20	8/30*=26.7%	3/13=23.1%	37.5 (11-65)	19.0 (12-30)
21-40	19/28 =67.9%	4/6 =66.7%	23.5 (8-49)	15.5 (13-18)
41-60	16/20 =80.0%	4/5 =80.0%	46.3 (6-124)	49.8 (11-139)
61-80	17/21 =81.0%	5/6 =86.7%	30.1 (8-121)	16.8 (8-32)

* The numerator shows the number of animals died of the tumor, and the denominator indicates the number of animals received tumor transplantations.

the transplantability showed a gradual increase with the passage of transplantation. Detailed data are given in Table 1. At the present time, both Wistar-King and Wistar rats are highly susceptible to the ascites hepatoma (H-17) which show a transplantability exceeding 80 per cent in the above two strains. On the other hand, the average survival time of tumor-bearing animals did not decrease with the increase of transfer generations. In the Wistar rats, the mean life spans were 37.5, 23.5, 46.3 and 30.1 days for the 1st to 20th, the 21st to 40th, the 41st to 60th and the 61st to 80th generations, respectively. Compared with that of the 1st 20 generations, the mean life span decreased in the 2nd 20 generations, whereas in the next 20 generations it showed an increase beyond that of the first 20 generations, but it again decreased in the 61st to 80th generations. A similar situation occurred in the Wistar-King rats (Table 1). As seen in Table 1, in the 41st to 80th generations, the life days of the tumor-animals showed a very wide range of variation. Some animals survived over 100 days and then died of tumor. At autopsy tumor invasions were rarely observed though there was a rich ascitic fluid of intense hemorrhage containing a small number of freely floating tumor islands.

In conclusion it can be stated that with the accretion of transplant generations the ascites hepatoma shows a gradual increase in transplantability. But there is no parallel relationship between the change in transplantability and in survival of tumor-bearing animals.

3. ***Chromosome pattern***: During a period from the first to 5th generations of serial transfers, the growth of this ascites hepatoma was very slow and therefore no sufficient material was obtainable for chromosome analysis. Moreover, a striking stickiness of chromosomes prevented the exact counting of their number. With the increase of transfer generations, the growth of this tumor became pronounced and the chromosomes came to show less stickiness. Chromosome counting was undertaken in 21 to 25 reliable metaphase plates in the samples taken from the 6th to 9th, the 19th to 25th, the 60th to 62nd, and the 74th to 78th generations, respectively (Text-fig. 2). In the samples derived from the 6th to 9th generations, the chromosome number varied from 66 to 81 without showing distinct modal values, being multi-modal in nature (Text-fig. 2). With transfer

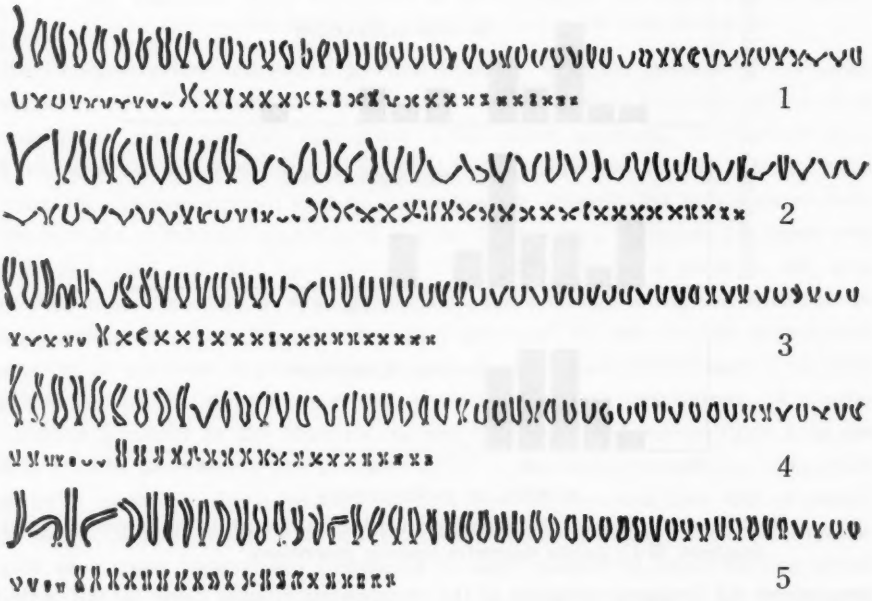


Text-figure 2. Changes in chromosome number distribution in the ascites hepatoma (H-17) during successive transfer generations.

generations the frequent variation of the chromosome number came to fall wthin a rather narrow range with conspicuous modal values. The variation ranges were 67 to 78 for the 19th to 25th generations, 67 to 74 for the 60th to 62nd, and 67 to 71 for the 74th to 78th generations, respectively. The most recent samples taken from the 74th to 78th generations showed the modal chromosome number

lying between 69 to 71 in over 80 per cent. Though the samples derived from the 60 th to 62 nd generations and from the 19 th to 25 th generations showed obscure modal values in chromosome number, they suggested the existence of the most common number lying between 69 and 71, as understood from the data in Text-figure 2. The cells having the modal chromosome number of 69 to 71 occupied 58 per cent in the samples taken from the 60 th to 62 nd generations, while 50 per cent in those from the 19 th to 25 th generations. In the samples derived from the 6 th to 9 th generations, however, only 12 per cent of cells observed showed 69 to 71 chromosomes.

The chromosome constitution of this tumor was provided with rod-like, V-shaped and J-shaped elements of varying size and number. In Figures 1 to 5 are presented five representative metaphase complexes observed in the samples taken from the 8 th, 20 th, 60 th, and 70 th transfer genrations. Comparison was made by means of the ideogram analysis in which the chromosomes were arranged in two groups i. e., those of a rod or J-shape and those of a V-shape. The chromosomes were arranged in each group in a roughly graduated order of size. Since the variation



Figures 1–5. Ideogram analyses of the ascites hepatoma (H-17). Fig. 1, 79 chromosomes from the 8 th generation. Fig. 2, 73 chromosomes from the 8 th generation. Fig. 3, 71 chromosomes from the 20 th generation. Fig. 4, 71 chromosomes from the 60 th generation. Fig. 5, 70 chromosomes from the 74 th generation.

in arm ratio of chromosomes is fairly continuous, a clear-cut demarkation between J- and V-shaped chromosomes is always difficult. Further the same trouble always occurs between the J-shaped chromosomes having an extremely short arm and telocentric rod-shaped ones. Accordingly the J- and rod-shaped elements were included in one group. After comparison, one can not detect any remarkable difference in the chromosome complex between the five ideograms shown in Figures 1 to 5, though there ocur some differences between them in total chromosome number. But it should be mentioned that morphological likeness of chromosomes does not always mean similarity in their inner structure since the chromosome of the tumor may undergo inner changes involving structural rearrangements and small adjustments that may correlate with the shift of the neoplastic condition occurring in successive transfers.

Discussion

The interest of the present author was focused upon some transitional changes of several neoplastic characters of a rat ascites hepatoma in its serial transfers.

Yoshida (1956, 1957) has reported several types of hepatoma islands in different kinds of ascites hepatomas of rats; they are large compact islands, large irregularly shaped ones, small uniformly-shaped ones, and samller and fewer islands containing isolated cells. In a mouse ascites hepatoma, Sato *et al.* (1956) have found free-cell sublines which arose spontaneously from the island-type hepatoma. But it is interesting to note that in the rat ascites hepatoma dealt with in this paper, a similar size-pattern of cell-islands was observed as a transitional type in successive transfers.

As described in the foregoing pages, the percentage of transplantability increased with the accretion of transplantations, though there is no parallelism between survival time of tumor-bearing animals and the change of transplantability. A similar feature was observed to occur in several ascites hepatomas which developed in non-inbred rats by Yoshida (1956). Those ascites hepatomas showed a wide range of variation in their survival time after transplantion either in non-inbred rats or in inbred rats (Sato, 1955). A similar tendency was also obtained in the present hepatoma which was induced in a highly purebred rat: it showed a wide survival range after transplantation either in rats of the same strain or in rats of different pure lines. In the early tarnsfer generations no purely inbred animals showed a high transplantability for this tumor. In the Novikoff rat hepatoma, Novikoff (1957) reported a fall of the life span of the tumor-animals after 6 months' successive transfers. According to Tanaka and Kanô (1952, 1954) their ascites hepatoma established in a pure rat was characterized by relatively constant life spans ranging from 15 to 20 days.

In earlier transplant generations, the chromosomes of this tumor varied very widely in number. But, the range of numerical variation became narrower and narrower with the increase of transfer generations. It is likely that some shifts occur in both chromosome constitution and number during serial transfers of this hepatoma. In the Yoshida sarcoma Makino (1957 b) and Makino and Sasaki (1958) observed a transitional increase in frequency of the tumor stem-cells in the course of successive tranfers. In view of the above evidence it is apparent that in earlier transfer generations, there are present several lines of stem-cells forming the neoplastic population and as a result there occurs a wide range of variations in chromosome number. With the increase of transfer generations the stemline-chromosomes may undergo certain numerical and structural alterations which might lead to genotypic changes of the neoplastic population. Then the cells, which have the most successful combination of chromomes capable of reproductive capacity on the one hand, and on the other hand which have obtained the most favorable adaptability in respect to the physiological condition of the host, may form distinct stemlines after several transfer generations of selection. In view of the above consideration, it is likely that the tumor cells having 69 to 71 chromosomes in this tumor may have attained the most adaptive genotypic adjustment favorable for proliferation in the host and then they may take a role as stem-cells. Makino (1957 c) reported that a hypotriploid stem-line of the MTK–IV tumor started from the hypotetraploid line through gradual elimination of certain chrmosomes during serial transfers. Tonomura and Sasaki (1957) reported that the stemline chromosomes of the MTK–sarcoma II and III had undergone morphological and numerical shifts during several year's serial transfers.

It can be concluded on the basis of th available data at hand that the constancy of the stem-line, while it is very pronounced, is not necessarily fully stable, and that under certain conditions the ascites tumors of rats have undergone numerical and structural changes of chromosomes during successive transfers. It is highly evident that such changes will cause transition in the genotype of the tumor which will produce shifts in the characters of the tumor. The evidence presented in this paper furnishes a fine example which supports the above view involving the evolutional alteration of the neoplastic conditions.

Summary

The present study deals with several transitional changes occurring in the neoplastic property of an azo-dye induced ascites hepatoma of the rat since its development. With the accretion of transplant generations, the hepatoma islands showed a gradual change in general structure. There occur also transitions in transplantability and life-span of the tumor-bearing animals during the course of

serial tranfers. In earlier transfer generations, the chromosome number of tumor cells of this hepatoma fluctuated within a very wide range, without showing any distinct modal values. With the increase of transfer generations, the chromosome number showed a rather narrow range of variation with the occurrence of distinct modal values at 69–71.

It can be can be concluded on the basis of the present findings that the constancy of the stem-line, while it is very pronounced, is not necessarily fully stable, and that under certain conditions the tumors have undergone numerical and structural changes in their stemline-chromosomes during serial transfers. Apparently such chromosomal changes will cause the transition of the genotype of the tumor which will in turn produce the shifts in the property of the tumor.

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Explanation of Plates XXVII and XXVIII

Figure 10. Histological feature of the original hepatoma; haematoxylin and eosin preparation, ×500.

Figures 6-9. Photomicrograghs of metaphase chromosomes of the ascites hepatoma (H-17). Fig. 6, 73 chromosomes from the 8th generation, ×1900. Fig. 7, 71 chromosomes from the 60th generation, ×2000. Figs. 8-9, 70 chromosomes from the 74th generation, 8, ×1900, 9, ×2000.

Figures 11-13. Ascites hepatoma (H-17), smear preparations stained with acetic dahlia, ×120. Fig. 11, primary tumor ascites. Fig. 12, 25th generation, 5 days after transplantation. Fig. 13, 67th generation, 5 days after transplantation.

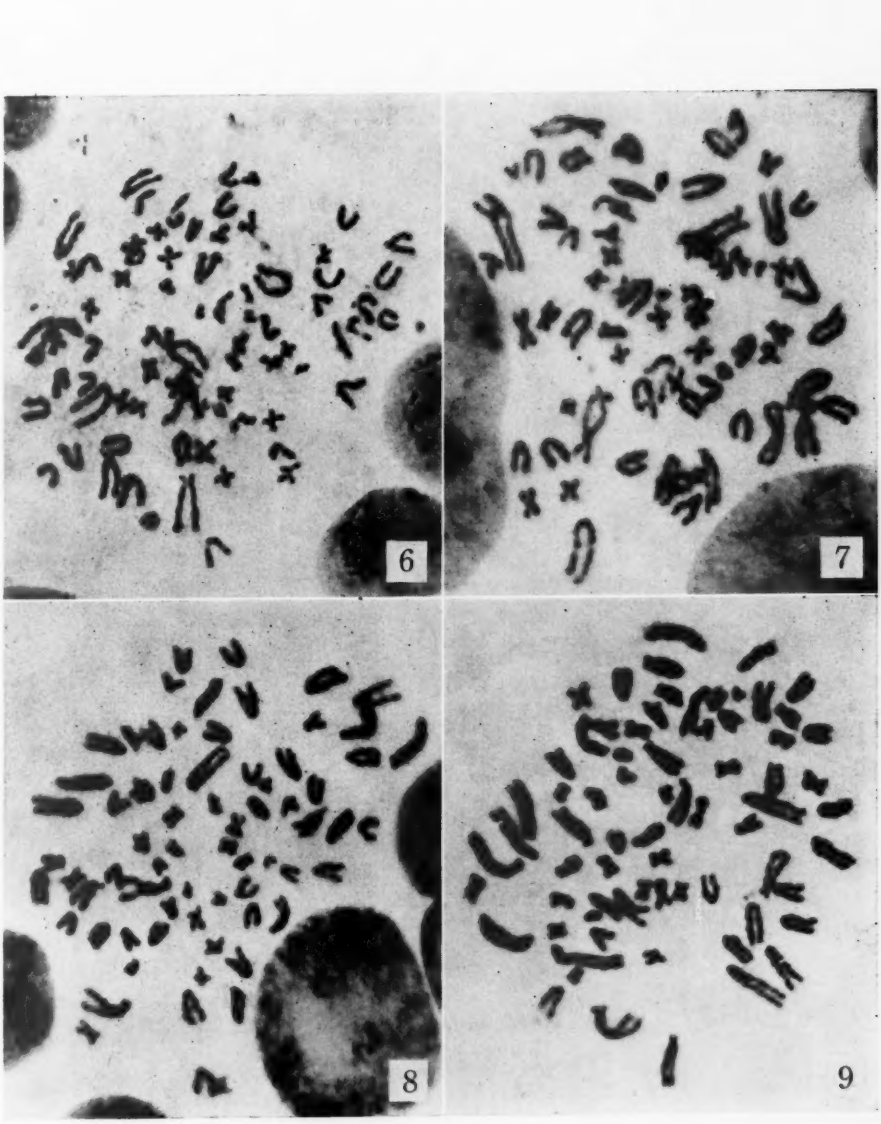
要　旨

癌の細胞学的研究，XXIV.
シロネズミ腹水肝癌 (H-17) の異代移植中にみられた腫瘍の性状の変化

佐々木本道
(北海道大学理学部動物学教室)

p-Dimethylaminoazobenzene 経口投与により高度の純系シロネズミ Wistar-King A 系 (F_{158}) に発生した腹水肝癌 (H-17) の累代移植中にみられた肝癌島の性状，移植性，生存日数，染色体数の変化について調査した。

約 90 代 (2 年間) の累代移植中にその移植率は次第に増加したが腫瘍動物の平均生存日数は必ずしも移植率の増大と平行して減少しなかった。また，肝癌島の性状も次第に変化し，その大きさは累代移植とともに小さくなった。一方，このような変化と平行して染色体数の変異の幅が次第に狭くなり高い mode を示すようになった。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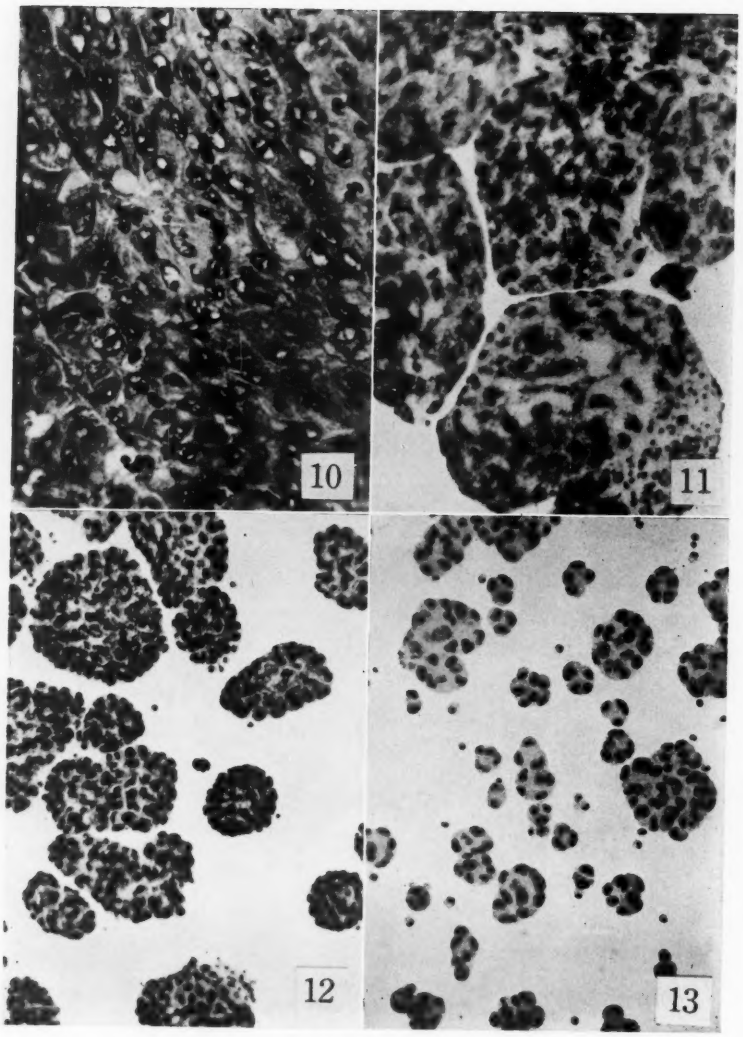
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